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IMMUNOLOGICAL STUDIES ON PURE CULTURES OF VARIOUS SPIROCHETES.

By HIDEYO NOGUCHI, M.D., and SEINAI AKATSU, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

As a sequel to the successful cultivation of *Treponema pallidum* and other allied forms of spirochetes,[1] an era of test-tube experimentation on the question of immunity in syphilis has been inaugurated. Previous to the time when these organisms were obtained in pure cultures, extensive experiments were carried out with suitable animals by various investigators, notably by Metchnikoff and Roux,[2] Neisser and his associates,[3] Landsteiner and Finger,[4] Uhlenhuth and Mulzer,[5] and others. Summing up their results, we are confronted with the fact that in syphilis no immunity, in the sense generally understood in bacterial infections, is demonstrable either in animals or in man.[6,7] The insusceptibility of animals or human beings who have once contracted the infection and have since been apparently cured of a subsequent infection with *Treponema pallidum*, a fact well recognized since the time of Ricord,[8] is no longer regarded as a state of immunity, but of an anergy,[3] which means that the same organism is not capable of responding to another infection as long as there is a preexisting infection. Moreover, clinical and experimental data show that when an individual is cured of an attack, he soon regains practically his original susceptibility to a second

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[1] Noguchi, H., *Münch. med. Woch.*, 1911, lviii, 1550; *J. Exp. Med.*, 1911, xiv, 99; 1912, xv, 81, 90, 466; xvi, 194.

[2] Metchnikoff, E., and Roux, E., *Ann. Inst. Pasteur*, 1903, xvii, 809; 1904, xviii, 1, 657; 1905, xix, 673; 1906, xx, 785.

[3] Neisser, A., Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911; also *Arb. k. Gsndhtsamte.*, 1911, xxxvii, 569; *Deutsch. med. Woch.*, 1906, xxxii, 1, 97.

[4] Landsteiner and Finger, *Centr. Bakteriol., 1te Abt., Ref.*, 1906, xxxviii, Beil., 107. Landsteiner, K., *Centr. Bakteriol., 1te Abt., Ref.*, 1908, xli, 785.

[5] Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte.*, 1913, xliv, 307.

[6] Zinsser, H., *J. Lab. and Clin. Med.*, 1916, i, 785.

[7] Levaditi, C., *Z. Immunitätsforsch., Ref.*, 1910, ii, 277–318.

[8] Ricord, P., Traité de la syphilis, Paris, 1845.

infection.[9] In this respect, syphilis bears more resemblance to a protozoan than to a bacterial disease. Attempts to study the immunity question *in vitro* have not been lacking, and numerous investigators claim to have observed diverse specific immunity reactions, such as precipitation,[10] immobilization,[11] complement fixation, [12, 13] etc., when the serum or cerebrospinal fluid had been brought in contact with a material containing the treponemata of syphilis. But analysis of these observations proved that many of these phenomena could not be confirmed, and none was strictly specific. The Wassermann reaction is very constant in syphilis, but not specific. It is evident, therefore, that there is no recognized specific antigen-antibody reaction in syphilis. Schereschewsky[14] tried agglutination tests with his impure cultures of a spirochete derived from syphilitic material, with inconclusive results. We cannot, however, accept the negative findings of earlier investigators as final until it has been demonstrated that the techniques employed cannot be further improved. The great obstacle in the way of satisfactorily testing immune reactions in syphilis lies in the fact that a sufficient quantity of virulent organisms free from tissue constituents is obtained only with difficulty. A pure culture would fill this requirement.

In 1912 Noguchi[15] had already commenced to employ his several strains of culture *pallidum* for the purpose of studying the various immunity problems which were awaiting solution by means of a culture material. The main efforts were directed to finding out whether a rabbit repeatedly inoculated with the pure *pallidum* cultures will become resistant to a subsequent inoculation with a virulent *pallidum*. The cultures used were already avirulent. For comparison, another series of rabbits was similarly treated with live and killed virulent *pallida* for the same length of time, which covered a period of 5 months. The mode of immunization consisted in intravenous and intratesticular inoculation, except in the case of the live virulent material, which could not be used intratesticularly on account of its tendency to start the infection in the organ. The aim of the intratesticular mode of immunization was to find out whether there is such a thing as a local immunity in syphilis. The immunized animals were tested with a virulent strain by inoculating it into their testes. It was found that six out of the twelve rabbits immunized with the culture *pallidum* intravenously took the inoculation, while five of the twelve rabbits receiving the virulent *pallidum* took. Those which were immunized with the *pallidum* emulsions (live culture and killed

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[9] John, F., *Samml. klin. Vortr.*, 1909, 559 ff.

[10] Fornet, W., *Berl. klin. Woch.*, 1908, xlv, 85.

[11] Hoffmann, E., and von Prowazek, S., *Centr. Bakteriol., Ite Abt., Orig.*, 1906, xli, 741, 817.

[12] Detre, L., *Wien. klin. Woch.*, 1906, xix, 619.

[13] Wassermann, A., Neisser, A., and Bruck, C., *Deutsch. med. Woch.*, 1906, xxxii, 745.

[14] Schereschewsky, *Deutsch. med. Woch.*, 1909, xxxv, 1652.

[15] Noguchi, *J. Am. Med. Assn.*, 1912, lviii, 1163.

virulent strains) lost much of the glandular structure of their testes, some becoming mere strands of hard connective tissue. Positive takes were recorded in three of the six rabbits treated with the culture and two of the six treated with the killed *pallidum* from a syphilitic orchitis of the rabbit. As the number of animals was so small it was impossible to draw any conclusion. The results as compared with control series with normal rabbits were striking, since the takes in the normal animals were practically 90 per cent. There was an indication that the repeated inoculation of the rabbit with the *pallidum* material reduced susceptibility to a certain extent, although the difference may have been due to the altered structure of the inoculated testes. The immune sera obtained from these rabbits were also tested for agglutination, spirochetolysis, complement deviation, and opsonic property against their homologous and cross antigens. The results were at first encouraging, but repeated experiments soon showed them to be indecisive on account of the technical difficulties in the way of obtaining satisfactory antigens. This was so even with the culture *pallidum*, which at that period was either very difficult to obtain free of culture media or underwent spontaneous agglomeration.

Craig and Nichols,[16] employing alcoholic extracts of pure cultures of the *pallidum*, *pertenuis*, and *microdentium*, furnished by Noguchi, reported that these antigens fixed complement with syphilitic sera very much as does an alcoholic extract of a congenitally luetic fetal liver.

Kolmer,[17] in the meanwhile, employing a strain of culture *pallidum* furnished by Noguchi, prepared immune sera in the rabbit and demonstrated the presence of agglutinins for the strain used. His most powerful serum agglutinated the *pallidum* in a dilution of 1 : 1,280. Kolmer, Williams, and Laubaugh[18] next studied a series of human and animal sera with regard to complement fixation, with the culture *pallidum* as antigen. Their positive findings, although more numerous, were nevertheless similar to those already obtained by Noguchi.[15] On the other hand, they noticed that rabbit immune sera not only fixed complement with their homologous antigens, but also with washed typhoid and cholera antigens. They point out, as was previously emphasized by Noguchi,[19] that these immune sera do not fix complement with the alcoholic extract of the culture *pallidum*.

Kissmeyer,[20] employing a strain of culture *pallidum* as antigen in the agglutination tests with human sera, obtained a consistent result when compared with the clinical and Wassermann reactions, but this apparent specificity was evidently

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[16] Craig, C. F., and Nichols, H. J., *J. Exp. Med.*, 1912, xvi, 336.

[17] Kolmer, J. A., *J. Exp. Med.*, 1913, xviii, 18.

[18] Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *J. Med. Research*, 1913, xxviii, 345.

[19] Noguchi, *J. Exp. Med.*, 1909, xi, 84.

[20] Kissmeyer, A., *Deutsch. med. Woch.*, 1915, xli, 306.

not confirmed by Zinsser, Hopkins, and McBurney.[21] The recent contributions made by these investigators[22] have advanced our knowledge of the immunity phenomena in connection with the cultivated avirulent and uncultivated virulent *pallidum* strains; comparing the behavior of both towards the immune sera prepared by means of the former. They came to the conclusion that the immune serum behaves in the same manner as a bacterial immune serum when tested upon the culture *pallidum*; that is, it produces agglutination, causes destruction of the organism with the cooperation of fresh complement, and fixes complement with the culture antigen. On the other hand, no immunity phenomena are demonstrated when the immune serum and the virulent uncultivated organisms of the same strain are brought together. They have not mentioned, however, whether or not an immune serum prepared by means of injecting the virulent material repeatedly will ever produce a protective or spirocheticidal principle.

The work of Kolmer and Zinsser, Hopkins, and McBurney, to which we have referred, has brought out a number of facts, particularly with regard to the relation between the avirulent culture and the virulent tissue *pallidum* strains, but the question is still far from being solved. It is not superfluous, therefore, that any data bearing upon this phase of the study should be published.

The present paper deals with the results of immunological studies which had been interrupted but were recently resumed. As will be seen from the following account, we have used a considerably larger number of culture spirochetes and of animals and continued the immunization longer than any worker has previously reported. We have purposely avoided touching the question of the relation between the cultivated and uncultivated strains, as we expect to consider that point in a future paper.

EXPERIMENTAL.

Material and Scope of Experiments.

In the present series of immunization experiments, rabbits of about 3 kilos were employed. Several rabbits were used for each of the four strains of *Treponema pallidum*, and several also for each of the following: *Treponema calligyrum*, *Spirochæta refringens*, *Treponema microdentium*, and *Treponema mucosum*. The mode of immunization

[21] Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, xxiv, 561.

[22] Zinsser, H., and Hopkins, J. G., *J. Exp. Med.*, 1915, xxi, 576; 1916, xxiii, 323. Zinsser, Hopkins, and McBurney, *ibid.*, 1916, xxiii, 341.

consisted in the intravenous injection every week of 1.5 to 3 cc. of the fluid culture of each strain, the strains having been grown in a medium consisting of equal parts of normal rabbit serum and faintly alkaline bouillon, with a piece of fresh rabbit kidney. The ascitic fluid usually used was replaced by rabbit serum in order to avoid the production of a specific precipitin or of complement-binding antibodies for human proteins in the animals immunized against the spirochetal cultures. The organisms grew fairly well in this medium, owing undoubtedly to their gradual adaptation previously to various culture media which originally were unsuitable for their growth. The age of the cultures varied from 14 to 30 days, and the number of spirochetes was approximately thirty to forty per field when examined under the dark-field microscope (Leitz oc. 3, $\frac{1}{12}$ oil immersion).

The aim of these immunization experiments was to study the development of such agglutinins, complement-binding antibodies, opsonins, and spirocheticidal principles as may be demonstrated *in vitro* in the sera of rabbits repeatedly inoculated with spirochetes. The immunity reactions were first tested with the specific organisms and then with those not used for the production of the immune sera in question. The cross examinations, various non-specific as well as

TABLE I.

Immunization Tests with Treponema pallidum.

Rabbit No.	*T. pallidum.*	Period of immunization.	No. of injections.
		1915	
1	Strain McD.	Sept. 14–Dec. 17	13
2	" "	" 14– " 17	13
3	" "	" 10– " 17	11
4	" "	Oct. 20– " 17	8
5	Strain XI.	Sept. 14–Dec. 17	13
6	" XI.	" 14– " 17	13
7	" XI.	" 30– " 17	11
8	" XI.	" 30– " 17	11
9	Strain B29.	Sept. 14–Dec. 17	13
10	" B29.	" 30– " 17	11
11	Strain Z. A.	Sept. 14–Dec. 17	13
12	" "	" 14– " 17	13

the specific antigens being used against a given immune serum, were undertaken with a view to establishing the relation which may exist between different members of the group of spirochetes.

The protocols of immunization are given in Table I.

The results of experiments with the immune sera may be summarized under separate heads.

Agglutinins.

The mode of determining the content of agglutinins in each serum was conducted in the following manner.

Into a series of small sterile test-tubes were measured quantities of the immune serum in amounts graduated from 0.1 to 0.000001 cc. for each tube. The distribution of the various amounts was carried out as is usual in such procedures; namely, by adequately diluting the serum with a 0.9 per cent saline solution and then taking out such quantities of each dilution as are required for titration. To each of the tubes containing various quantities of the serum was added 0.1 cc. of the spirochetal emulsion (as antigen), and the final volume was brought up to 1.5 cc. by adding the necessary quantities of 0.9 per cent saline solution to each tube. The content of the tubes was thoroughly mixed by shaking and the tubes were placed in a water incubator at 37°C. At least two tubes containing the spirochetal emulsion alone were prepared at the same time and served as controls. Concentrated and washed suspensions of various spirochetes derived from pure cultures in a fluid medium were used as antigens. 0.1 cc. of the suspension of each strain in 0.9 per cent saline solution was used for each tube. The number of spirochetes varied in different suspensions, but there were over 100 per field (Leitz oc. 3, $\frac{1}{12}$ oil immersion, dark-field illumination), and the addition of 0.1 cc. of the antigen emulsion to each tube (total volume of fluid, 1.5 cc.) produced grayish white, semitranslucent turbidity. The turbidity in the control tubes gradually subsided while standing in the incubator, but was never completely cleared up, even after 24 hours' standing. Readings of the results were made twice, once after 2 hours' incubation, and again at the end of 24 hours at a temperature of 15°C. The sera were used without any modification, such as inactivation at 56°C.

As may be seen from Table II, we first tested the agglutinating powers of various immune sera for their homologous strains; that is, for the strains which had been used for producing them in the rabbits by repeated intravenous injections. Thus, eleven sera were titrated with four different strains of the *pallidum*, two to four immune sera for each strain. As in the case of the *pallidum* the remaining

immune sera, eight in number, were tested with their homologous species, the *calligyrum*, *refringens*, *microdentium*, and *mucosum*, which had been employed for their production.

It may be mentioned that we had to make a number of preliminary experiments before we felt assured of obtaining fairly uniform and reliable results. The reading of a strong agglutination was quite easy, as the organisms rapidly settled down, and the sediment adhered firmly to the side and bottom of the tube, but the less intense reaction was not as clear as we wished; hence, our reading of the minimum zone of the reaction was more or less arbitrary. The titers of agglutinins in different sera read after 2 hours' incubation were somewhat lower than those recorded after the same set of tubes had been left at room temperature for 24 hours longer. It was always necessary, in order to detect a slight degree of agglutination, to shake up the sediment and compare the granulation or clumping of the suspension with a control tube without any serum. We have resorted solely to the macroscopic reading of the reaction. A microscopic examination of the sediment adhering rather firmly to the side and bottom of the test-tubes where a definite agglutination occurred revealed enormous masses of entangled spirochetes, some apparently undergoing morphological modifications, as shown by a granular appearance or by relaxation of curves.

In Table II several facts seem to stand out conspicuously. In the first place, the titers of agglutinins developed in the rabbits treated with the saprophytic species, namely, the *calligyrum*, *refringens*, *microdentium*, and *mucosum*, are decidedly lower than those found in the sera produced by immunizing the rabbits with the *pallidum*. Secondly, the amounts of agglutinins in different immune sera are not in direct proportion to the number of injections or the duration of the immunization. Thus, the highest titer was found in Serum 4, in which 0.00001 cc. caused a definite, and 0.000025 cc. a slight agglutination, while Serum 9, notwithstanding the fact that the animal had five more injections than the former, required 0.0025 cc. for a definite reaction. Again, it is evident, with regard to the production of the agglutinins, that different rabbits react differently to the same spirochetal antigens. The apparent difference between the titers of the *pallidum* group and those of the other group requires an ex-

TABLE II.

Tubes Incubated for 2 Hours in the Water Bath. Antigen: 0.1 Cc. of Emulsion of Fluid Cultures 2 Months Old. Total Volume 1.5 Cc.

Antigens.	Immune sera.	Titers of agglutinins.
		cc.
T. pallidum, Strain McD	No. 2	0.000025
	" 3	0.0001
	" 4	0.00000375
" XI	" 5	0.00005
	" 6	0.0005
	" 7	0.00004
	" 8	0.000025
" B29	" 9	0.001
	" 10	0.00005
" Z. A	" 11	0.001
	" 12	0.000025
T. calligyrum	" 13	0.0005
	" 14	0.001
S. refringens	" 15	0.00125
	" 17	0.001
T. microdentium	" 18	0.0025
	" 19	0.001
T. mucosum	" 20	0.001
	" 21	0.01

planation. Whether the phenomenon is due to the greater amounts of agglutinins produced in the rabbits immunized with the *pallidum* or to a physical or possibly also chemical factor inherent in the *pallidum* antigen has not been established. It is also possible that our results with the saprophytic varieties happened to be inferior merely because the animals used were unfavorable individuals. The results might have been different if we had employed more animals.

Of more interest are the results of cross examinations of various immune sera with regard to their specificity towards the homologous and heterologous antigens. Eight immune sera were chosen to be tested, each with six strains of *Treponema pallidum* and one of *Treponema calligyrum, Spirochæta refringens, Treponema microdentium,* and *Treponema mucosum,* or ten different antigens in all. Stated in more detail, the procedure was as follows:

Serum 4 was produced by injecting the animal with Strain McD. of the *pallidum* and was tested not only with the emulsion of the same strain (homologous), but also with those of the Strains XI, R, C_2, B30, and Z. A. of the *pallidum*, as well as with those of the *calligyrum*, *refringens*, *microdentium* and *mucosum* (heterologous and of different species). Serum 8, produced with Strain XI, and Serum 12, produced with Strain Z. A. of the *pallidum*, were likewise tested against their homologous as well as their heterologous antigens, while Serum 10, the homologous antigen of which was Strain B29, was tested with all ten heterologous antigens, since there was no homologous one in the series. Sera 14 and 17 of the *calligyrum* and *refringens* of the non-pathogenic spirochetes of the genitalia, and Sera 18 and 20 of the *microdentium* and *mucosum* of the buccal cavity were each tested with one homologous and nine heterologous antigens. In order to secure as closely comparable results as possible, all the tests were carried out in parallel series on the same day and with the same materials. The first reading was recorded after completion of the 2 hour incubation in the water thermostat and the second at the end of the 24 hour period at room temperature. The latter readings are given in Table III.

In the analysis of Table III, several points are brought out. In the first place, there is a pronounced degree of specificity of each characteristic for its homologous group of antigens. Thus, the immune sera belonging to the *pallidum* group agglutinated most strongly when brought together with the emulsion of the *pallidum* strains, but not at all when mixed with the *microdentium* or *mucosum*. The reverse is also true; that is, the immune serum produced by means of the *microdentium* agglutinated none of the other groups, except for a slight reaction with the *mucosum*. The *mucosum* immune serum showed its strongest action on the *mucosum* antigen, although there was a more or less feeble reaction with some of the other spirochetes, especially the *microdentium*. The relation between the *calligyrum* and *refringens* seems very close, considered from the point of view of the agglutination reaction. They did agglutinate mutually to such an extent that they might be included in the same group. As already mentioned above, they showed no affinity whatever for the group of the mouth spirochetes. On the other hand, there existed a certain degree of so called group reaction between the *pallidum* and the *calligyrum* groups. It may be pointed out that in spite of the close relation between the *calligyrum* and the *refringens*, the immune sera pertaining to the *pallidum* did not noticeably agglutinate the *refringens*, while the same sera invariably agglutinated the *calligyrum*

TABLE III.

Specificity of Immune Sera towards the Homologous and Heterologous Antigens. Results of Incubation for 24 Hours at Room Temperature.

Antigens.	Immune sera.							
	No. 4 homologous with Strain McD.	No. 8 homologous with Strain XI.	No. 10 homologous with Strain B29.	No. 12 homologous with Strain Z. A.	No. 14 homologous with *T. calligyrum.*	No. 17 homologous with *S. refringens.*	No. 18 homologous with *T. microdentium.*	No. 20 homologous with *T. mucosum.*
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
T. pallidum.								
Strain McD.....	0.0005	0.0005	0.0005	0.0025	0.05	Trace at 0.05.	None at 0.05.	None at 0.05.
" XI.....	0.0005	0.0005	0.0005	0.005	0.05	Trace at 0.05.	None at 0.05.	None at 0.05.
" R.....	0.0005	0.005	0.005	0.005	0.05	0.05	None at 0.05.	Trace at 0.05.
" C_2.....	0.0005	0.001	0.0005	0.001	0.01	Trace at 0.05.	None at 0.05.	Trace at 0.05.
" B30.....	0.0005	0.0025	0.0005	0.005	0.05	Trace at 0.05.	None at 0.05.	Trace at 0.05.
" Z. A.....	0.0005	0.0005	0.0005	0.0005	0.05	0.05	None at 0.05.	Trace at 0.05.
T. calligyrum.....	0.05	0.05	0.025	0.025	0.0005	0.0075	None at 0.05.	Trace at 0.05.
S. refringens.....	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	0.005	0.0025	None at 0.05.	Trace at 0.05.
T. microdentium...	None at 0 05.	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	None.	0.001	0.01
T. mucosum.....	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	"	0.05	0.001

in doses between 0.05 and 0.025 cc., that is, in a dilution of 1:20 to 1:40. This group reaction is, of course, far below the titers of these sera for the *pallidum* antigens, in which they varied from 0.005 (1:200) to 0.0005 cc. (1:2,000), and still further down to 0.00001 cc. (1:100,000) (Table II). Conversely, the *calligyrum* serum, which agglutinated its own antigen in a dose of 0.0005 cc. (1:2,000) produced a group reaction with the *pallidum* emulsions in doses ranging from

0.05 (1:20) to 0.01 cc. (1:100). In this respect, the behavior of the *refringens* serum was somewhat similar to that of the *calligyrum*, as it also produced a distinct agglutination with some of the *pallidum* antigens when employed in a concentration stronger than 1:20, sometimes even in a dilution of 1:40.

As to the results obtained among the *pallidum* antigens with the corresponding and cross immune sera, it will be noticed that the reaction between the homologous antigens and immune sera are, as a rule, stronger than those which occurred when the former were mixed with the sera produced by the other strains. On the other hand, a serum which strongly agglutinates a certain strain or strains may not necessarily agglutinate others, while the latter may yet be the most readily and strongly agglutinated by another serum. In other words, our present study indicates that the agglutinin titers of these sera are variable according to the differences in the individual strains of the same group. This phenomenon has long been recognized in the agglutination of various bacteria by their immune sera, and apparently it holds good in the case of the spirochetes. The complex composition of agglutinins, such as partial agglutinins of different affinities or still only scantily understood factors in agglutination reactions, is accountable for the intricacy of the so called specific as well as group reactions.

Influence of Time upon the Agglutinins in Vitro and in Vivo.

The immune sera used in the foregoing experiments were preserved in a refrigerator at the temperature of 6°C. for 3 months and then examined for their strength. In the meantime, the rabbits immunized were not given any further injection of the spirochetal emulsions for the same length of time, and then their sera were drawn for the titration of agglutinins. Table IV shows the titers of these sera as compared with their original strength.

The rates with which the agglutinin contents of the immune sera lost strength during the 3 months seem to be irregular and show no constant proportion to the original titers of the sera. There is, however, a general tendency of the agglutinins to disappear from the serum more rapidly *in vivo* than *in vitro*. The titers of the ag-

TABLE IV.

Effect of Time upon Agglutinins in Vitro and in Vivo.

Immune sera.	Titer.		
	Original.	After 3 mos. kept at 6°C.	After 3 mos. in the animal.
	cc.	*cc.*	*cc.*
No. 2, *T. pallidum*, Strain McD	0.000025	0.00025	0.001
" 8, *T.* " " XI	0.000025	0.0005	0.001
" 10, *T.* " " B29	0.00005	0.0001	0.001
" 12, *T.* " " Z. A	0.000025	0.0001	0.001
" 14, *T. calligyrun*	0.001	0.01	0.1
" 15, *S. refringens*	0.00125	0.01	0.1
" 19, *T. microdentium*	0.001	0.01	<0.1
" 20, *T. mucosum*	0.001	0.01	<0.1

glutinin in the sera derived from these rabbits at the end of 3 months after discontinuation of immunization were reduced to about one-one hundredth in Nos. 14, 15, 19, 20; one-fortieth in Nos. 2, 8, 12; and one-twentieth in No. 10, while the original sera when preserved in the refrigerator during the same period of time lost their strength down to about one-tenth of the initial titers, except in No. 8, where it was almost twice as much weakened as in the rest. It may therefore be assumed that during the first 3 months after the cessation of immunization, the disappearance of the agglutinins for various spirochetes was almost ten times as fast in the animal body as in the test-tubes kept at a temperature of 6°C.

Complement Fixation.

The immune sera were also tested for their property of binding complement when mixed with their homologous as well as heterologous antigens. The technique of carrying out the tests was that usually employed in such experiments.

The immune sera were inactivated at 56°C. for half an hour before use. The antigens were prepared from the pure cultures of various spirochetes grown in a fluid medium consisting of equal parts of rabbit serum and slightly alkaline bouillon, with a piece of normal rabbit kidney. The spirochetes were collected from cultures which had been vigorously growing for 4 weeks. For the purpose of concentration and purification, the spirochetes were collected by a prolonged

centrifugation and washed again in 0.9 per cent saline solution. The sediment, resuspended in an adequate volume of 0.9 per cent saline solution, showing about twenty to thirty spirochetes under dark-field illumination, was used as the antigen. The relative quantities of different factors were as follows: The immune serum in quantities of from 0.1 to 0.0001 cc., complement 0.04 cc. (2 units), antigen 0.2 cc., sheep corpuscles 5 per cent, anti-sheep amboceptor 0.0002 cc. (corresponding to 2 units), total volume made to 1.5 cc. with 0.9 per cent saline solution. The first incubation was for 1 hour (at 36°C. in the water bath), and the second also for 1 hour. The quantities of immune serum and the spirochetal suspensions chosen for the above experiments were previously tested and found to be not anticomplementary in themselves. The results were recorded after allowing the tubes to stand for 2 hours longer at room temperature.

TABLE V.

Complement Fixation with Homologous Antigens.

Antigens 0.2 cc.	Immune sera.	Titers of fixation.
		cc.
T. pallidum.		
Strain McD	No. 2	0.005
	" 3	0.002
	" 4	0.005
" XI	" 5	0.005
	" 6	0.1
	" 7	0.005
	" 8	0.008
" B29	" 9	0.007
	" 10	0.003
" Z. A.	" 11	0.002
	" 12	0.0003
T. calligyrum	" 14	0.01
S. refringens	" 15	0.1
	" 17	0.03
T. microdentium	" 18	0.007
	" 19	0.05
T. mucosum	" 20	0.002
	" 21	0.005

Table V shows that the titers of the immune sera derived from different rabbits vary considerably according to the individual variations that exist among the animals. For example, Immune Sera 6 and 15 showed an extremely low power for complement fixation, in spite of the fact that the rabbits in the same groups, particularly

in the Strain XI series, produced fairly high titers. The titers of the *pallidum* group were in the main quite high, while the *calligyrum* and *microdentium* remained much less developed. It is interesting to note that of the two animals treated with the *mucosum*, both responded with high titers. When compared with the agglutinin titers of the same sera, the fixation titers are much lower but seem to run fairly parallel, in the sense that a serum with stronger agglutinating power had also a higher titer for complement fixation. There were a few exceptions to this rule.

For determining the question of specificity of the complement fixation reaction with the spirochetes a series of experiments was performed in which a certain number of the immune sera were tested, each in turn, with the entire set of antigens. Table VI gives the results. The technique used was the same as before.

Table VI shows that various immune sera fixed complement more strongly with their corresponding antigens than with a heterologous one. Among the *pallidum* strains, the reaction is fairly interchangeable, although some immune sera (Nos. 3 and 7) have caused only a weak fixation with a certain antigen (Strain B29). The *calligyrum* serum produced a marked fixation, not only with its own antigen, but also with certain *pallidum* strains (Strains XI and Z. A.) to such an extent that in a dilution of 1: 30 no difference could be distinguished between the *calligyrum* and these two *pallidum* strains. In this connection, mention may be made of an analogous instance where one of the *pallidum* immune sera (No. 3) fixed complement with the *calligyrum* antigen distinctly in a dilution of 1: 30. The *refringens* serum fixed complement best with its own antigen, but also feebly with all the other antigens except that of the *mucosum*. A similar group reaction was evident with the *calligyrum* and *mucosum* sera. In spite of the presence of a certain amount of group reaction, the specific character of the complement fixation phenomenon seems to hold. At least, the differences between the *pallidum*, *refringens*, *microdentium*, and *mucosum* are sufficient for considering the reaction specific. The *calligyrum* showed much affinity for the *pallidum* on the one hand and for the *refringens* on the other.

TABLE VI.

Complement Fixation with Heterologous Antigens.

Immune sera.		Antigens used for cross titration.							
		T. pallidum strain.				*T. calligyrum.*	*S. refringens.*	*T. microdentium.*	*T. mucosum.*
		McD.	XI.	B29.	Z. A.				
	cc.								
No. 3 homologous	0.1	++	++	<+	++	+	−	−	<<+
with Strain McD.	0.03	++	++	−	++	<+	−	−	−
	0.01	+	+	−	++	−	−	−	−
No. 7 homologous	0.1	+	++	++	++	+	−	−	−
with Strain XI.	0.03	+	++	<+	+	−	−	−	−
	0.01	<+	+	−	<+	−	−	−	−
No. 10 homologous	0.1	++	++	++	++	<+	−	−	−
with Strain B29.	0.03	++	++	++	++	−	−	−	−
	0.01	++	+	++	++	−	−	−	−
No. 11 homologous	0.1	+	++	++	++	<+	<+	−	−
with Strain Z. A.	0.03	<+	+	+	++	−	−	−	−
	0.01	−	<+	−	++	−	−	−	−
No. 14 homologous	0.1	<<+	++	<+	+	++	<+	<<+	<<+
with *T. calligyrum.*	0.03	−	<+	−	<+	<+	−	−	−
	0.01	−	−	−	−	<<+	−	−	−
No. 17 homologous	0.1	<+	+	<+	<+	+	+	<<+	−
with *S. refringens.*	0.03	−	−	−	−	<<+	<<+	−	−
	0.01	−	−	−	−	−	−	−	−
No. 18 homologous	0.1	−	−	−	<+	<<+	−	++	<+
with *T. microdentium.*	0.03	−	−	−	−	−	−	++	−
	0.01	−	−	−	−	−	−	<+	−
No. 21 homologous	0.1	<+	<+	<+	<+	<<+	−	<+	++
with *T. mucosum.*	0.03	−	−	−	−	−	−	−	++
	0.01	−	−	−	−	−	−	−	++

Spirocheticidal Properties.

In order to determine whether or not these immune sera have a destructive power upon the spirochetes *in vitro*, several series of tests were carried out. Because of the part played by complement in a bacteriolytic process, our experiments were so arranged that in one set the immune serum was allowed to act alone, while in another, both complement and immune serum were put together with the spirochetes.

The immune sera were inactivated at 56°C. for 30 minutes before use, and the spirochetes were from recent fluid cultures and were very active. The amount of immune serum was graduated from 0.1 down to 0.00005 cc., while spirochetes and complement were used in a quantity of 0.1 cc. each for each test-tube. The volume of the resulting fluid in each tube was made uniformly 1.1 cc. by adding 0.9 per cent saline solution. After all the factors, namely, spirochetes, immune serum, and complement, had been mixed, the tubes were placed in a water bath incubator at 37°C. for 1 hour, and then the whole contents were used for cultivation in the usual ascitic agar tissue medium. The results were recorded at the end of 2 or 3 weeks of incubation at 36°C. Normal rabbit serum was also tested as control.

The results of experiments in which most of the antisera were tested show that the anti-*pallidum* Serum 12 suppressed the growth of its homologous strain Z. A. in a dose of 0.001 cc. in the presence of complement, while scanty growth was observed in all the tubes where there was no complement added to the immune serum. Normal rabbit serum had no appreciable effect on the growth of this strain. It was noted also that the number of spirochetal colonies diminished almost proportionately with the gradual increase of the specific immune sera, until, in quantities of serum beyond a certain limit, no growth was observed. With the anti-*pallidum* Serum 4, and possibly also No. 8, the titer was 0.0003 cc. It was not rare for the anti-*pallidum* sera to attain the titer of 0.001 cc. On the other hand, the immune sera for the *refringens*, *calligyrum*, and *mucosum* were far inferior in their restraining influence upon their homologous species, none of them being strong enough to inhibit the growth in a dose below 0.01 cc. In the *mucosum*, 0.01 cc. of the antiserum failed to kill the organism.

In parallel series of experiments without the addition of complement, it was found that the powerful immune sera (Nos. 4 and 8) caused a considerable restraint upon the growth of the homologous strains, the only difference between these sera and those containing complement being that their effects are less marked. When used in quantities below 0.03 cc., neither of these sera caused a complete suppression of the growth of organisms acted upon by them. The question naturally presents itself as to whether the suppression of growth in cases where complement and the immune sera were employed was due to the actual destruction of the organisms or merely

to the agglutination. The latter assumption may be dismissed, as the phenomenon does not occur when the immune sera alone (containing the agglutinins) are used. Of course, the sparseness of colonies in these tubes must be partly due to the agglutination, which tends to clump many thousand spirochetes to start a single colony. Yet a total suppression cannot be explained on this ground, since a single colony, once it has begun to grow, will finally form a diffuse growth throughout the medium. As will be described later, the failure of the spirochetes to grow after being subjected to the action of complement and immune serum was largely due to the actual destruction of the spirochetes. Syphilitic rabbit serum was also tested and showed that there was an unmistakable destruction of the *pallidum* strain in quantities above 0.03 cc. Normal rabbit serum showed a slight restraint upon growth of this strain when used in doses of 0.1 cc. Neither of these sera had any ill effect upon the spirochetes when complement was omitted from the mixture.

Microscopic Observations on the Effect of the Immune Sera upon Spirochetes in Vitro.

Immune Sera 4 (Strain McD.), 8 (Strain XI), 10 (Strain B29), and 12 (Strain Z. A.) were chosen for a series of observations in which we followed the changes which take place in *Treponema pallidum* when the organisms have been subjected to the action of these sera. Three strains of the *pallidum*, McD., R, and Z. A., were used for the purpose. As in the previous experiments, the action of the immune sera was studied with and without the addition of complement (guinea pig fresh serum).

The sera were inactivated at 56°C. for 30 minutes. The mixture of the serum, spirochetal suspension (McD.), and complement (in cases where it was added) was incubated in a water bath at 37°C. for 3 hours, and microscopic examinations were made during and after incubation. Similar results were obtained with the strains of R and Z. A.

Table VII demonstrates the fact that *Treponema pallidum* undergoes a fundamental change in its morphology when acted upon by its immune sera and complement at an optimum temperature. The phenomenon is similar to the dissolution or disintegration of various

TABLE VII.

Effect of Immune Sera upon Spirochetes in Vitro.

Strain McD. 0.1 cc. + Immune Serum 4 (Strain McD.) 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + Immune Serum 8 (Strain XI) 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + Immune Serum 10 (Strain B29) 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + Immune Serum 12 (Strain Z. A.) 0.1 cc. + complement 0.1 cc.	The organisms were strongly agglutinated within 1 hr., then they became granular in appearance, and only a few retained their form.
Strain McD. 0.1 cc. + Immune Serum 4 (Strain McD.) 0.1 cc. + no complement. Strain McD. 0.1 cc. + Immune Serum 8 (Strain XI) 0.1 cc. + no complement. Strain McD. 0.1 cc. + Immune Serum 10 (Strain B29) 0.1 cc. + no complement. Strain McD. 0.1 cc. + Immune Serum 12 (Strain Z. A.) 0.1 cc. + no complement.	The agglutination was even stronger than in the above series. At the end of 24 hrs. the organisms kept their form.
Strain McD. 0 + no serum + complement. " " 0 + " " + no "	No noticeable changes.

microorganisms under the influence of an immune serum. In the absence of complement, the organisms were strongly agglutinated, but not dissolved, even after 24 hours.

In the following experiment, attempts were made to study the part, if any, played by leukocytes in the destruction of the spirochetes *in vitro*. Leukocytes both from normal and from immunized rabbits were collected by Wright's method. The citrate blood was briefly centrifuged, and the leukocytic layer of the sediment carefully skimmed and put into another centrifuge tube. In ordinary opsonin work, the cells thus collected are used without washing, but in the present experiment, they were washed with a 0.9 per cent saline solution by a renewed centrifugalization. The immune leukocytes were obtained from a rabbit which had been immunized with the Z. A. strain (No. 12).

The technique was as follows: To 0.1 cc. of a suspension of one of the *pallidum* strains were added 0.1 cc. of the leukocytic suspension, 0.1 cc. of each of the immune sera, and 0.1 cc. of complement. The mixtures, after being well stirred,

were incubated in a water bath at 37°C. and examined at various intervals; for example, 30 minutes, 1 hour, 2 hours, and 4 hours (kept at room temperature). The examinations were made without staining under the dark-field microscope, and also as film preparations stained by Giemsa as well as by a modified Fontana silver impregnation. Films prepared from a mixture in which unwashed citrate leukocytic suspension was used washed off the slides very readily and could not be depended upon for accurate results. Normal as well as immune serum and normal leukocytes were also used (Table VIII).

TABLE VIII.

Effect of Leukocytes upon Spirochetes in Vitro.

Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 4, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 8, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 10, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 12, 0.1 cc. + complement 0.1 cc.	Strong agglutination and gradual lysis, many spirochetes adhering to the leukocytes, into which some were definitely ingested. Total disappearance of spirochetes in 24 hrs.
Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 4, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 8, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 10, 0.1 cc. + complement 0.1 cc. Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 12, 0.1 cc. + complement 0.1 cc.	Strong agglutination and general disintegration of spirochetes but decidedly less than in the preceding series. Phagocytosis present.
Strain McD. 0.1 cc. + no leukocytes + Immune Serum 4, 0.1 cc. + complement 0.1 cc.	Much lysis in 24 hrs.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + no immune serum + complement 0.1 cc.	No striking changes.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + no immune serum + no complement.	No lysis or phagocytosis.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + normal serum 0.1 cc. + complement 0.1 cc.	No lysis or phagocytosis.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + normal serum 0.1 cc. + no complement.	No lysis or phagocytosis.

Parallel series of experiments were also carried out with Strains R and Z. A. with similar results. It seems apparent, therefore, that some of the spirochetes are readily taken up by immune as well as normal rabbit leukocytes, when an immune serum and complement are simultaneously added to the mixture. The number of spirochetes taken up by phagocytes is small compared with what we are accustomed to see with bacteria. It may be due to the filamentous feature of the organisms, which prevents the cells engorging them with readiness, or what appears still more probable is that they form enormous masses of entangled nets too large to be taken up by the phagocytes, and then gradual lysis occurs. It is also possible that the engorged spirochetes quickly disappear through intracellular digestion. At all events, the agglutinated masses become gradually granular and indistinct and finally undergo dissolution. The completeness with which this process proceeds is most marked when immune leukocytes are used. Perhaps there exists within these cells certain elements which render the lytic processes more energetic than when only immune serum and complement are used. In the absence of complement, the immune serum produced only a slight degree of phagocytosis and no lysis. The organisms, after being acted upon by the leukocytes in the immune serum and complement mixture, lose their property to take Giemsa stain as strongly as those not so treated. Their curves are seen to be flattened in many specimens. They still retain their affinity for the silver precipitation method (Fontana). It has been difficult to stain the spirochetes distinctly within the phagocytes, although they could be seen in fresh preparations by means of dark-field illumination. The large number of spirochetes adhering to the leukocytes prevents a clear appearance of the phenomenon.

A series of experiments was performed with normal human leukocytes in conjunction with the immune rabbit sera and complement. The results were similar to those recorded for normal rabbit leukocytes.

Effects of Immune Sera and Leukocytes upon the Uncultivated Strain of Treponema pallidum.

The action of the sera derived from rabbits immunized with cultivated strains of *Treponema pallidum* may also be studied. It may be tested by subjecting a suspension of a virulent testicular material rich in the *pallidum* to the effect of the immune sera and then inoculating certain animals susceptible to experimental syphilis. Another procedure would be to study the immunity phenomena which follow the mixing of the *pallidum* and the serum *in vitro*. Agglutination, immobilization, granular disintegration, dissolution, or complement binding may be studied. The first method is subject to the difficulties inherent in experiments in which the susceptibility of different individuals constitutes an inconstant factor. In fact, our preliminary experiments indicated that study with extensive material is required. This phase of the study has been under investigation by one of us since 1911 and will have to be continued for a longer time. The test-tube phenomena were better observed.

To 0.1 cc. of a rich suspension of virulent *pallidum* from syphilitic orchitis of a rabbit, 0.1 cc. of each of the several immune sera, comprising Nos. 4 (Strain McD.), 8 (Strain XI), 12 (Strain Z. A.), and 10 (Strain B29) was added, and 0.1 cc. of a 40 per cent solution of fresh guinea pig serum as complement. The mixture was made up to 1 cc. by adding a 0.9 per cent sterile saline solution and then placed in a water bath kept at the temperature of 37°C. The mixture, an opalescent fluid, was examined from time to time for the agglutination, motility, or disintegration of the *pallidum* under the dark-field microscope. Controls with the *pallidum* alone, the *pallidum* with the immune serum (inactivated), and the *pallidum* with the complement were provided.

The results of our observations were in the main similar to those obtained by Zinsser, Hopkins, and McBurney, except for a few points. It was noticed in our experiments that the particular strain of *Treponema pallidum* employed became sooner or later immobilized by the addition of the immune sera, and none was motile after 3 hours at 37°C. In the control tubes containing plain saline or complement solution, the organisms were still active for several hours longer. In some control tubes, there were a few motile *pallida* at the end of 24 hours. The immobilizing effect was augmented by the presence of complement. There was no definite agglutination or dissolution of the *pallidum* except in cases of Sera 4 and 8, where there were small clumps of entangled immobilized organisms suggesting a slight agglutination. But we were unable to find definite disintegration of the *pallidum*.

Studies were also made to discover whether or not leukocytes from an immune or normal rabbit ingested the tissue *pallidum* under the influence of the immune serum, but the results were inconstant. Although there was an unmistakable phagocytosis in the presence of the immune serum, it was slight in comparison with the cultivated strains, and there was no general disintegration of the organisms. In considering the above results, it appears as though the immune sera prepared by injecting the rabbit with avirulent strains of culture *pallidum* exerted much less effect upon a virulent strain derived from a syphiloma in a rabbit. Whether or not this is due to unsuitable immunization or to a modification in strains is not shown by the present experiments. The failure of agglutination of the tissue *pallidum* may have been due to the presence of various tissue proteins simultaneously introduced into the mixture, for these apparently indifferent substances can often interfere with agglutination or complement fixation.[23] The entire question as to the relation between the uncultivated virulent strains and those which had become avirulent through cultivation is still under investigation.

CONCLUSIONS.

Experiments were carried out for the study of culture spirochetes in their relation to various immunity reactions *in vitro*. Several strains of *Treponema pallidum* and one each of *Treponema calligyrum*, *Spirochæta refringens*, *Treponema microdentium*, and *Treponema mucosum* were used. Tests were made of immune substances responsible for agglutination, complement fixation, spirocheticidosis, and opsonization. In cases of agglutination and complement fixation, cross titrations were made.

1. In the sera derived from rabbits immunized with various spirochetes agglutinins were demonstrated in varying quantities for the homologous antigens. The amounts of agglutinins developed were considerably higher in the *pallidum* immune sera than in the other groups. There was no parallelism between the amounts of antigens injected and the amounts of agglutinins developed.

2. Cross titrations among different *pallidum* strains revealed that

[23] Noguchi, H., and Bronfenbrenner, J., *J. Exp. Med.*, 1911, xiii, 92.

the agglutination is not necessarily strongest when homologous antigens and immune sera are brought together.

3. On the other hand, the reactions between the immune sera and antigens belonging to different species were sufficiently specific to justify the grouping.

4. Certain degrees of group reaction were observed between the *pallidum* immune sera and the *calligyrum*, and occasionally very faintly also between the *pallidum* and the *refringens* antigens and *vice versa*. There was a much more pronounced group reaction between the *calligyrum* and *refringens*. The immune serum and antigen of the *microdentium* showed a slight affinity for the *mucosum* but none for the *pallidum*, *calligyrum*, or *refringens*, while the *mucosum* immune serum caused a slight agglutination with many members of the other groups. Hence, it appears that the *pallidum* is more or less related to the *calligyrum*, while the affinity between the *calligyrum* and *refringens*, and possibly also between the *calligyrum* and *mucosum* in a much smaller degree, seems close. The *microdentium* showed the least relation to any other spirochetes.

5. Titration of agglutinins in the sera obtained 3 months after the cessation of immunization revealed that the agglutinin contents were already greatly reduced, having fallen roughly to 0.01 of the original strength. The rates of disappearance were irregular in different animals and bore no direct relation to the initial titers. Titration made of the immune sera which had been preserved aseptically in a refrigerator (6°C.) during the same period (3 months) indicated that the original strength of these sera was reduced to about one-tenth. The agglutinins for spirochetes disappear from the rabbit's body much more rapidly than they are reduced in the separated sera by deterioration on standing at 6°C.

6. Titration of the immune sera for complement fixation power showed with a few exceptions, in which there was only slight complement binding, that the titers were high enough to indicate the presence of this principle. The anti-*pallidum* sera possessed higher average titers than the other immune sera tested with correspondingly homologous antigens. The least active were the anti-*refringens* sera.

7. Cross titration of anti-*pallidum* immune sera for complement fixation showed that a given serum with a high titer for its own strain of antigen was also strong with most of the other strains of the

pallidum. Instances occurred also in which the titers with heterologous *pallidum* antigens fell far below those of the homologous. Group reactions between the different spirochetes, such as the *pallidum* and the *calligyrum*, the *calligyrum* and the *refringens*, and the *microdentium* and the *mucosum*, were also indicated. The *mucosum* and the *pallidum* showed a slight degree of group reaction. No anti-*pallidum* serum fixed complement with the *microdentium*.

8. The immune sera were tested for their spirocheticidal properties *in vitro* against the correspondingly specific and heterologous varieties with and without the addition of complement. Many of the anti-*pallidum* sera killed their own strains. Normal rabbit serum exhibited only a slight degree of inhibition. Without complement, the immune sera caused a considerable reduction in the number or density of colonies, but not a complete suppression of growth. Complement alone had no injurious effect upon the *pallidum* strains. The antisera for the *calligyrum*, *refringens*, and *mucosum* showed feeble spirocheticidal action, while the antisera for *microdentium* was stronger. A syphilitic rabbit serum tested against a strain of culture *pallidum* gave a feeble inhibitory effect.

9. Under the influence of immune sera and complement, the spirochetes undergo within a few hours complete disintegration or granular degeneration. Without complement, they are more powerfully agglutinated, but no disintegration occurs, even after 20 hours, and complement alone has no effect.

10. In the presence of homologous immune serum and complement, the culture *pallidum* may be ingested by the leukocytes, but phagocytosis is slight, possibly on account of the filamentous nature of the organisms. The spirochetes in such a mixture disintegrate within a few hours, disintegration being especially rapid when the immune leukocytes are used. In the absence of immune serum, phagocytosis is not noticeable, while without complement but in the presence of immune serum and leukocytes, some phagocytosis, without subsequent lysis, occurs.

A virulent strain of *pallidum*, obtained from syphilitic orchitis in a rabbit, exposed to agglutination, lysis, and phagocytosis by an immune serum prepared by means of culture *pallidum* strains, showed only slight agglutination and phagocytosis but rapid immobilization without disintegration in the presence of complement.

A CONTRIBUTION TO THE BACTERIOLOGY OF ACUTE ANTERIOR POLIOMYELITIS.*

By JOHN A. KOLMER, M.D., CLAUDE P. BROWN, M.D., and ANNA M. FREESE, M.D.

(*From the Laboratory of the Philadelphia Hospital for Contagious Diseases and the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, Philadelphia.*)

Plate 66.

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During the recent outbreak of acute anterior poliomyelitis in Philadelphia (1916) we began a series of bacteriological studies with the blood and cerebrospinal fluid collected from patients during life and with various tissues secured at eight autopsies.

Early in the work we obtained cultures of diplococci, streptococci, and other microorganisms, but on account of the consensus of opinion to the effect that these bacteria are of little or no importance in the etiology of the disease, we devoted our efforts toward the cultivation of the organism described by Flexner and Noguchi (1) in 1913. As the result of recent communications by Mathers (2), Rosenow and his associates (3), and Nuzum and Herzog (4), setting forth renewed claims for the etiologic relation of these cocci to acute anterior poliomyelitis, we have given the cultures further and more extensive study, and the purpose of this communication is to present the results of our work with many different cultures of these easily cultivated bacteria isolated from persons ill with acute anterior poliomyelitis.

* Part of the paper was read in the symposium upon acute anterior poliomyelitis before the Philadelphia County Medical Society, November 8, 1916. This work was instituted in the laboratory of the Philadelphia Hospital for Cutaneous Diseases and continued in the laboratories of Pathology of the University of Pennsylvania, with the cooperation of Dr. Allen J. Smith, Dr. Charles K. Mills, and others, as a part of a series of investigations bearing upon the microparasitology and clinical aspects of poliomyelitis.

It is probable that the micrococci recently described and regarded as the specific etiologic agent of acute anterior poliomyelitis were met with early in the course of investigations bearing upon the etiology of this disease. Bülow-Hansen and Harbitz (5) and Harbitz and Scheel (6, 7) found a diplococcus in the cerebrospinal fluid of several cases of acute anterior poliomyelitis and referred to the work of Geirsvold (8), who found a diplococcus in the cerebrospinal fluid of twelve cases and claimed to have produced paralysis and death in experimental animals with them. Pasteur, Foulerton, and Maccormac (9) reported the discovery of a micrococcus in the cerebrospinal fluid of a case during life, which produced in rabbits symptoms resembling the disease in human subjects. Leiner and von Wiesner (10) and Krause and Meinicke (11) reported that these micrococci were not the etiologic agents of acute poliomyelitis, as had Flexner and Lewis, who first showed that the etiologic agent was filterable through dense filters and probably belonged to the filterable viruses (12); simultaneous and similar results were observed and reported by Landsteiner and Levaditi (13). Dixon, Fox, and Rucker (14) also found a diplococcus in the cerebrospinal fluid, nose, and throat of patients with acute anterior poliomyelitis, and Rucker made one of the fullest studies of this diplococcus apparently identical with that recently described by Mathers (2) and Nuzum and Herzog (4). While the latter have reported the successful infection of various laboratory animals with these cocci and the reproduction of a disease with clinical symptoms and lesions similar to those of acute poliomyelitis, the experiments of the former were negative throughout; an inoculated monkey succumbed with a hemorrhagic meningitis, but without clinical or histological evidences of anterior poliomyelitis.

Rosenow (3) has recovered what he calls a "peculiar polymorphous streptococcus" from the tonsils, brain, cord, mesenteric lymph glands, and once from the blood, but never from the cerebrospinal fluid of cases of acute poliomyelitis, which produced lesions and symptoms among the lower animals regarded as those of acute poliomyelitis. He has described changes in size and staining reaction of these streptococci according to the culture medium employed, the age of the culture, and whether they have been grown aerobically or anaerobically. The cocci were said to become very small under anaerobic conditions and to approach in size the globoid bodies described by Flexner and Noguchi; the small forms were found to be filterable through Berkefeld filters while the larger forms were not.

We have found various microorganisms and especially a diplococcus and a streptococcus not only in the cerebrospinal fluid but also in the tissues of the central nervous system and in various other internal organs of fatal cases; in no instance, however, have we been able to produce anterior poliomyelitis in monkeys and rabbits with any culture by intracranial, intravenous, or intraperitoneal injection of these microorganisms.

Microorganisms Isolated.

From the cerebrospinal fluid of poliomyelitic patients during life and from various tissues after death, we have cultivated anaerobically four different varieties of microorganisms as follows: (*a*) streptococci, (*b*) diplococci, (*c*) diphtheroids, and (*d*) Gram-negative bacilli.

As stated above, we have divided the micrococci into two groups; namely, those which grow out into long chains and present the characteristics of streptococci and those which assume a diplococcus arrangement in small clumps and short chains. Apparently Rosenow believes that these are the same microorganism with a polymorphous nature. Before summarizing the results of our anaerobic cultures of the cerebrospinal fluid, blood, and tissues of poliomyelitic patients, the different microorganisms which we have recovered may be briefly described.

Streptococcus.

1. In cultures of tissues containing streptococci in an ascites-broth-kidney medium under sterile paraffin oil, macroscopic growth was first apparent after 5 to 9 days' incubation at 37°C.

2. Primary anaerobic and aerobic cultures and particularly subcultures of the streptococci grew in the form of a granular sediment at the bottom and sides of the tubes with a clear or but slightly cloudy supernatant medium. The diplococci, however, grew more diffusely.

3. Cultures under paraffin oil and in a Novy jar in an atmosphere of hydrogen grew somewhat more slowly than cultures under oil only.

4. Aerobic transplants from the anaerobic cultures to suitable solid or fluid media presented a visible growth within 48 hours.

5. Aerobic cultures of the streptococcus were readily secured by culturing emulsions of tissue in ascitic broth at 37°C. Anaerobic cultures required more time (at least 5 to 9 days, as stated above).

6. Films of 5 to 9 day anaerobic cultures showed short chains of Gram-positive cocci; longer chains were found later, although they may be found in the initial growth. As a rule, these cocci were round or slightly flattened; in a few instances the cocci in young anaerobic cultures have been elongated, while in older cultures the spherical shape was noted.

7. On defibrinated horse blood agar the colonies were small and of three varieties: (1) those which were non-adherent and produced a greenish pigment; (2) those which were non-adherent and hemolytic; and (3) those which were non-adherent and non-hemolytic.

8. Definite capsules were never found with any culture even after passage through mice, although films of cultures in ascitic broth stained after the method of Gram not infrequently showed slight unstained halos about the cocci suggestive of capsules.

9. In older anaerobic cultures the streptococci became smaller; after 14 days' incubation at 37°C. the majority of cocci were much smaller than those in fresh anaerobic and aerobic cultures. Large and small forms have been found together and the small cocci are easily decolorized during staining after the method of Gram.

10. Of sixteen cultures studied in relation to acid production with various carbohydrates, two were found to produce acid with inulin.

11. Aerobic and anaerobic cultures of streptococci were obtained from the cerebrum, cerebellum, pons, and cord after remaining in a 50 per cent mixture of pure neutral glycerol and sterile salt solution for periods of time varying from 10 days to 8 weeks.

The notable features of these streptococci were their slow multiplication in anaerobic cultures; the more rigid the anaerobiosis, the slower were the growths. The cocci became progressively smaller under anaerobic conditions, more easily decolorized with alcohol in the Gram method of staining, and more indefinite in outline as viewed microscopically.

Compared with films of mass cultures of the microparasite described by Flexner and Noguchi stained after the method of Gram but without counterstaining, our cocci were somewhat larger. The organism of Flexner and Noguchi occurs in short chains and irregular clumps, and while in our older anaerobic cultures we could find occasional cocci comparable with them in their minute size, yet the majority of our preparations showed uniformly distinctly larger forms.

Diplococci.

The most frequently cultivated microorganism in the cerebrospinal fluid and tissues of acute anterior poliomyelitis and apparently that first described occurs in the form of a Gram-positive diplococcus arranged in isolated pairs, tetrads, short chains, and irregular clumps. This diplococcus seems neither to have been named nor has its relation to known diplococci been established. The important morphological and biological characters of our cultures of these diplococci correspond closely with those described by various investigators.

1. In anaerobic cultures in ascites-broth-kidney medium of cerebrospinal fluid and emulsions of tissue containing this microorganism growth first appeared after 3 days' incubation at 37°C. Aerobic cultures presented macroscopic growth in 24 hours. In fluid medium the growths were diffuse with the gradual formation of a slimy sediment along the sides and bottom of the tubes.

2. Subcultures of the anaerobic cultures on ordinary culture media grew luxuriantly, producing whitish moist colonies resembling *Staphylococcus albus*.

3. Films of the aerobic and young anaerobic cultures showed a Gram-positive coccus usually arranged in diplococcus formation in chains of four or five pairs and in small clumps. Films of older anaerobic cultures (14 days or more) showed that the majority of these cocci had become smaller and many were easily decolorized by alcohol in the Gram stain.

4. Aerobic subcultures usually showed a staphylococcus grouping with a loss of the diplococcus formation. On solid media the tendency for the formation of short chains was lost.

As with the streptococci these diplococci tend to become smaller under strict anaerobic conditions as do cultures of *Staphylococcus albus* under similar conditions. These diplococci have many properties in common with varieties of diplococci found in the skin by one of us (15), and except for the reason that they have been found in tissues and cerebrospinal fluid collected under apparently aseptic conditions, we should be strongly inclined to regard them as microorganisms derived from the skin and mucous membranes.

Diphtheroid Bacilli.

These bacilli, which were short, solid, or occasionally granular in appearance and Gram-positive, did not present any difficulties in their recognition. They grew slowly under anaerobic conditions, but luxuriantly on appropriate media under aerobic conditions, producing large whitish colonies resembling the pseudodiphtheria bacillus rather than the true virulent bacillus. They were non-virulent for animals and their biological properties were those commonly found with cultures of diphtheroids recovered from enlarged lymphatic glands and mucous membranes.

Gram-Negative Bacilli.

In anaerobic and aerobic cultures of emulsions of poliomyelitic tissues and also of cerebrospinal fluid during life, two varieties of Gram-negative bacilli were found. (1) The usual variety was a short bacillus which morphologically and biologically appeared to be a member of the *B. coli* group. The majority produced gas with dextrose, produced indole, and coagulated milk. (2) In a few cultures

of the tissues a second Gram-negative bacillus was found which was much smaller than the preceding and resembled the influenza bacillus. Further statements regarding these bacilli cannot be made at this time except to state that we do not regard them as *B. influenzæ*.

Results of Anaerobic Cultures of Cerebrospinal Fluid in Acute Anterior Poliomyelitis.

Anaerobic cultures were made of the cerebrospinal fluid from 106 cases of acute anterior poliomyelitis by placing 1 or 2 cc. of the freshly drawn fluid collected under aseptic precautions, in tubes of ascitic fluid, or ascitic fluid and broth containing sterile rabbit kidney, and covered with sterile paraffin oil. After incubation for 5 days or more the cultures were examined and studied; in Table I is given a summary of the results of these cultures, showing the different bacteria found. Not infrequently a culture of one fluid contained two or even three different microorganisms.

The majority of these cultures were made within a few hours after the admission of the patients to the Philadelphia Hospital for Contagious Diseases and at varying intervals after the onset of symptoms.

TABLE I.

Anaerobic Cultures of the Cerebrospinal Fluid of 106 Cases of Acute Anterior Poliomyelitis.

Microorganism.	Cultures showing presence of microorganism.
Sterile	16
Streptococci	None.
Diplococci	48
Gram-negative bacilli	22
Diphtheroids	22
B. subtilis	20

As shown in this table, definite streptococci were not found; the diplococcus was found in forty-eight fluids, or 45 per cent of those cultured. As previously stated, this microorganism may occur in pairs or short chains in the anaerobic cultures, but aerobically and on solid medium a staphylococcus arrangement is usual.

Gram-negative bacilli and diphtheroids were found in 20 per cent of cultures. *Bacillus subtilis* was also found in 19 per cent. This microorganism is of course a contamination, and not only indicates the difficulties of avoiding contamination in the collection of spinal fluids from a large number of small, sick children, but casts doubt on the series as a whole (16).[1]

Results of Anaerobic Cultures of Various Tissues in Acute Anterior Poliomyelitis.

Anaerobic cultures of various tissues from fatal cases of poliomyelitis were made during or immediately after autopsy, in ascites-kidney and ascites-broth-kidney media. All the cultures reported herein were cultivated under paraffin oil only and not in the Novy jar. The tissues removed at autopsy were collected with care to exclude bacterial contamination as much as possible, but the conditions were not such as always to accomplish this end. Hence the portion of tissue selected for culture was dipped momentarily in boiling salt solution or water before being cultured, obviously an imperfect method of securing even superficial sterilization of the tissues. Here again, as Table II shows, the variety of bacterial forms cultivated indicates that either postmortem or agonal invasion had taken place or unavoidable contamination had occurred during removal of the organs.

As shown in Table II, streptococci were found in the brain and cord of at least 50 per cent of our cases. The presence of streptococci in various other internal organs including the thymus gland, liver, spleen, mesenteric glands, kidneys, suprarenal glands, and pancreas was also noteworthy. While a streptococcus has been reported in the lymphatic glands, yet its widespread distribution in the tissues of poliomyelitic patients has not been determined heretofore, as indicated in our studies.

[1] The experience of the Department of Health of the City of New York with cultures of the cerebrospinal fluid is valuable in this connection (16). One difference in the results may be attributable to the fact that we employed the anaerobic method of cultivation which develops growth under conditions in which the aerobic cultures do not.

TABLE II.

Results of Anaerobic Cultures of Various Tissues in Fatal Cases of Anterior Poliomyelitis.

Organ.	Total No. examined.	Results of cultures.				
		Sterile.	Streptococci.	Diplococci (staphylococci).	Diphtheroids.	Gram-negative bacilli.
Cerebrum	8	2	4	3	0	3
Cerebellum	8	1	4	2	0	2
Pons and medulla	7	2	6	4	0	2
Cord	8	1	4	4	1	3
Exudate on cord	6	0	5	2	2	1
Thymus gland	2	0	2	0	0	0
Lungs	3	0	3	2	0	0
Liver	3	1	1	2	0	0
Spleen	3	1	3	1	0	0
Kidneys	3	0	2	1	1	0
Mesenteric glands	4	0	3	3	0	1
Pancreas	4	2	1	1	0	0
Suprarenals	2	1	1	0	0	0
Tonsils	2	0	2	2	1	0

The streptococci from the internal organs resembled those from the brain and cord, except that the former showed a tendency to grow in longer chains.

The diplococci were likewise found not only in the brain and cord but also in various internal organs.

The diphtheroids were found infrequently, while Gram-negative bacilli were found mostly in the cultures of the brain and cord.

Anaerobic Cultures of the Blood in Acute Anterior Poliomyelitis.

Blood cultures were made of twenty cases of anterior poliomyelitis during the acute stages of the disease by withdrawing 1 to 3 cc. of blood from a vein at the elbow under aseptic precautions and culturing in ascites-broth-kidney medium under paraffin oil. A streptococcus was recovered in one instance, while a staphylococcus was secured in ten. The latter in the fluid medium had a diplococcus arrangement while transplants on solid medium such as plain or glucose agar yielded profuse whitish colonies resembling *Staphylococcus*

albus. These cocci were practically indistinguishable from the micrococci cultivated from the spinal fluid. Since the blood has been cultured repeatedly without result, the question arises again whether contamination was not also the explanation of the presence of diplococci in the blood.

Filtration Experiments.[2]

Since Flexner and Lewis demonstrated that the etiologic agent of acute anterior poliomyelitis is filterable through filters which hold back such microorganisms as *Micrococcus prodigiosus*, filtration experiments are of considerable interest and importance in the study of the etiology of this disease. The later experiments of Flexner and Noguchi showed that the minute globoid bodies were filterable, and Rosenow has reported that the small forms of the polymorphous streptococcus are filterable through Berkefeld N filters.

We have conducted a number of experiments with salt solution emulsions of tissues containing diplococci and streptococci and with various pure cultures of these and other microorganisms. In the absence of reliable Berkefeld filters we have employed the small Kitasato (fine) and larger Pasteur-Chamberland (fine) candles. The tissues had been in 50 per cent glycerol and salt solution for varying intervals of time, but cultures of the emulsions made at the time of the filtrations showed the presence of viable microorganisms.

Pieces of tissue about the size of a bean were washed several times in sterile water and ground in sterile mortars with sterile sand, with the addition of about 15 cc. of sterile salt solution. The emulsion was then centrifuged or filtered through sterile paper and anaerobic cultures were prepared. The emulsions were then passed through the various sterilized filters with the aid of a suction pump and anaerobic cultures made of the first 3 cc. of filtrate and of larger amounts of filtrate

Pure cultures of various microorganisms were first subcultured anaerobically and then passed through the candles and cultured in the same manner.

The results of these experiments are summarized in Tables III and IV.

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[2] We are indebted to Dr. Bertha M. Meine for assistance in conducting a number of these experiments.

TABLE III.

Results of Filtration Experiments with Emulsions of Poliomyelitic Tissues.

Material (glycerolated tissues).	Microorganisms present.	Filter used.	Amount of filtrate cultured.*	Results of anaerobic cultures of filtrates.†
Emulsion of cord (M).	Diplococci, streptococci.	Kitasato.	First 3 cc.	Sterile.
" " " (K).	Diplococci, streptococci, Gram-negative bacilli.	"	" 3 "	"
" " " (K).	Diplococci, streptococci, Gram-negative bacilli.	"	3 cc. of 15 cc. of filtrate.	Diplococci, streptococci, Gram-negative bacilli.
" " " (H).	Diplococci.	Pasteur-Chamberland.	First 3 cc.	Sterile.
" " " (C).	Streptococci, diplococci.	Pasteur-Chamberland.	" 3 "	"
" " " (C).	Streptococci, diplococci.	Pasteur-Chamberland.	3 cc. of 12 cc. of filtrate.	"
" " cerebrum(M).	Diplococci.	Kitasato.	First 3 cc.	"
" " " (H).	"	"	" 3 "	"
" " " (H).	"	Pasteur-Chamberland.	" 3 "	"
" " pons(M).	Streptococci (pure culture).	Kitasato.	" 3 "	"
" " " (M).	Streptococci (pure culture).	Pasteur-Chamberland.	" 3 "	"
" " " (K).	Streptococci, diplococci.	Kitasato.	3 cc. of 16 cc. of filtrate.	Growth of streptococci and diplococci.
" " " (K).	Streptococci, diplococci.	Pasteur-Chamberland.	3 cc. of 16 cc. of filtrate.	Sterile.

* Cultures in ascites-broth-kidney medium under paraffin oil.
† After 28 days' incubation at 37°C.

As shown in Table III, the filtrate of the emulsions of tissues with both the small Kitasato and larger Pasteur-Chamberland filters was invariably sterile if the first 3 cc. or less of filtrate were cultured; when larger amounts of emulsion were filtered and cultured, growths

TABLE IV.

Results of Filtration Experiments with Pure Cultures of Microorganisms from Poliomyelitic Tissues.

Culture* (acites-broth-kidney medium).	Microorganisms present.	Filter used.	Amount of filtrate cultured.†	Results of anaerobic cultures of filtrates.‡
5 day anaerobic.	Streptococci (large forms).	Kitasato.	First 3 cc.	Sterile.
5 " "	Streptococci (large forms).	"	3 cc. of 10 cc. of filtrate.	"
7 " "	Streptococci (mostly large forms).	Pasteur-Chamberland.	First 5 cc.	"
14 " "	Streptococci (many small forms present).	Kitasato.	" 3 "	"
14 " "	Streptococci (many small forms present).	"	3 cc. of 17 cc. of filtrate.	Streptococci.
17 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	First 4 cc.	Sterile.
17 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	4 cc. of 19 cc. of filtrate.	"
19 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	5 cc. of 31 cc. of filtrate.	Streptococci.
8 " "	Diplococci.	Kitasato.	First 3 cc.	Sterile.
8 " "	"	"	3 cc. of 12 cc. of filtrate.	"
8 " "	Gram-negative bacilli.	"	First 3 cc.	"
8 " "	" "	"	3 cc. of 11 cc. of filtrate.	"

* Subcultures of all of these in ascites-broth-kidney medium preliminary to filtration showed the presence of viable microorganisms.

† In ascites-broth-kidney medium under paraffin oil.

‡ After incubation for 11 to 28 days at 37°C.

were sometimes secured with the Kitasato filtrates. The amount of filtrate used in these experiments is therefore of considerable importance.

Experiments with pure cultures of various microorganisms showed

in general that larger amounts of filtrate may contain microorganisms, while the first few cubic centimeters may be sterile; also that streptococci in older anaerobic cultures (containing many small forms) may be filterable, while younger cultures, containing mostly larger forms, are not. There is no doubt that the streptococci and also the diplococci secured by us from poliomyelitic tissues tend to become much smaller when grown in fluid medium under paraffin oil, and as shown in the above experiments these small forms may be filterable under conditions which hold back larger forms. Of even more importance, however, is the amount of culture passed through a candle, as the passage of large amounts may wash through a number of the microorganisms.

Results of Animal Inoculation Tests.

We have injected cultures of the various microorganisms into rabbits, and a few cultures of streptococci and diplococci into monkeys intracranially, intravenously, and intraperitoneally. All the animals were carefully observed for clinical evidences of poliomyelitis and after death sections were prepared of the brain and cord and examined for histological evidences.

Rabbit Inoculation Experiments.—The cultures injected into rabbits were derived from the following sources.

No. of cultures.	Cultures.	Source.
14	Streptococci.	Poliomyelitic cords.
5	"	" brains.
2	"	Spleen.
3	"	Mesenteric glands.
5	Diplococci.	Cerebrospinal fluids.
3	"	Poliomyelitic cords.
2	"	" brains.
4	Diphtheroids.	" tissues.
3	"	Cerebrospinal fluids.
6	Gram-negative bacilli.*	Poliomyelitic tissues.

* These bacilli were not of the colon group.

With the exception of two cultures all were transplants from the original anaerobic ascites-broth-kidney cultures, in order to eliminate

to a large extent the possible injection of original tissue present in the primary culture and to insure the injection of pure cultures. The aerobic cultures used for injection had been incubated at 37°C. for 24 hours, and in all instances stained films showed the presence of numerous microorganisms. The doses administered are given in Table V. The animals varied in weight from 1,400 to 1,600 gm.

TABLE V.

Results of Rabbit Inoculation Tests.

Microorganism.	No. of cultures injected.	Route of inoculation.	Dose.	Results.
			cc.	
Streptococci.	18	Intracranial.	0.5–0.8	No paralyses; six deaths 4, 18, 20, 21, 26, and 26 days later.
"	24	Intravenous.	1.0*	No paralyses; one arthritis; seven deaths 1, 2, 9, 15, 19, 35, and 56 days later.
"	24	Intraperitoneal.	2.0*	No paralyses; three deaths 3, 7, and 10 days later.
Diplococci.	7	Intracranial.	0.5–0.6	No paralyses; one death 28 days later.
"	10	Intravenous.	1.0	No paralyses; one death 3 days later.
"	10	Intraperitoneal.	2.0	No paralyses; no deaths.
Diphtheroids.	4	Intracranial.	0.5–0.6	No paralyses; one death 36 days later.
"	7	Intravenous.	1.0	No paralyses; one death 34 days later.
"	7	Intraperitoneal.	2.0	No paralyses; one death 36 days later.
Gram-negative bacilli.	3	Intracranial.	0.5–0.6	No paralyses; three deaths 11, 14, and 21 days later.
" "	6	Intravenous.	1.0	No paralyses; three deaths 1, 25, and 35 days later.
" "	6	Intraperitoneal.	2.0	No paralyses; one death 28 days later.

* Dose per kilo of body weight.

As shown in Table V, which summarizes the number of different cultures of various microorganisms injected in different routes, true clinical and histological evidence of poliomyelitis was not observed in a single instance.

The streptococci produced arthritis in one rabbit and the intracranial injections were followed in several animals by a slight meningitis with recovery of streptococci at autopsy.

The streptococci caused more deaths than the diplococci, diphtheroids, and Gram-negative bacilli.

The intravenous injections were followed in several instances by the development of fatal pleuritis and pericarditis, from which lesions and from the blood of the heart, streptococci were recovered. On the other hand, the deaths of many of these animals and particularly those succumbing 3 and 4 weeks or longer after injection were due to secondary and unrelated causes.

In several of the animals injected with the diphtheroids and Gram-negative bacilli and succumbing 2 weeks or more later, these microorganisms were not recovered, whereas cultures of the heart and various internal organs frequently showed the presence of streptococci, indicating that the animals are subject to independent and secondary fatal infections with streptococci.

Monkey Inoculation Experiments.—The inoculation experiments have been limited to four animals. The intracerebral injection of the recently cultivated diplococci failed to produce anterior poliomyelitis (No. 1); likewise the intracerebral injection of five different strains of streptococci failed, producing, however, in one case a meningitis (No. 2); likewise the intravenous (No. 3) and intraperitoneal (No. 4) injections of streptococci failed to produce symptoms or lesions of poliomyelitis. The brief protocols of these four experiments are as follows:

Monkey 1.—Macacus rhesus; inoculated intracerebrally under ether anesthesia with 0.8 cc. of a mixture of the first anaerobic ascitic broth subcultures of recently glycerolated cords of four cases of poliomyelitis. The cultures were incubated for 4 days and showed numerous diplococci arranged in pairs and short chains. The animal promptly recovered from the anesthetic and operation, showed a slight disinclination to move about for 3 days, but has never showed any weakness or paralysis (44 days' observation). Subcultures were employed to aid in the elim-

ination of the injection of original tissue; the diplococci were, therefore, 8 days in artificial medium before inoculation.

Monkey 2.—Macacus rhesus; inoculated intracerebrally under ether anesthesia with 0.8 cc. of a mixture of 3 day aerobic ascitic broth cultures of five different strains of streptococci recovered from two cords, one cerebrum, one spleen, and one thymus gland of five different cases of poliomyelitis. Two strains were in the first transfer, and the remaining three were in the third transfer from the original anaerobic cultures. The animal promptly recovered from the anesthetic and operation and developed a severe meningitis lasting over 2 weeks, with pus and streptococci in the cerebrospinal fluid. The animal did not move about or use its hind legs, but repeated tests failed to discover paralysis.

Monkey 3.—Cebus capucinus; inoculated intravenously with 5 cc. of a mixture of 4 day aerobic ascitic broth cultures of five different strains of streptococci in the second and third transfers from original anaerobic ascites-kidney-broth cultures of three cords, one pons, and one spleen of five different cases of poliomyelitis. This animal presented a mild rise in temperature over a period of 2 days following the injection, with no other disturbances and no weakness or paralysis (56 days' observations).

Monkey 4.—Cebus capucinus; inoculated intraperitoneally with 5 cc. of the same mixture of five different strains of streptococci given to Monkey 3. This animal has not shown weakness or paralysis (56 days' observations).

In considering this series of experiments on monkeys note should be taken of the fact that they reproduce chiefly the method of inoculation which succeeds with the filtered poliomyelitic virus. In one instance only was an intravenous injection given and in it the dose was below that employed by Rosenow. However, the large quantity of cultures inoculated should have sufficed to develop symptoms of poliomyelitis had the streptococci used possessed the power of inducing that disease.

DISCUSSION AND SUMMARY.

Four different varieties of easily cultivated microorganisms have been cultured from the cerebrospinal fluid and tissues of cases of acute anterior poliomyelitis; namely, a streptococcus, a diplococcus, diphtheroids, and Gram-negative bacilli. It is not contended that they were all inherent in the tissues; a part were doubtless extraneous.

The streptococci and diplococci may be considered as the most significant of the bacteria cultivated and are distinguishable from each other by biological tests.

The streptococci grew both aerobically and anaerobically; under anaerobic conditions growth was slow, the cocci became small and round, and were more easily decolorized with alcohol in the Gram stain. They were not found in the anaerobic cultures of 106 cerebrospinal fluids; they were found in one of twenty anaerobic blood cultures and frequently in the cerebrum, cerebellum, pons and medulla, cord, tonsils, lungs, liver, kidneys, spleen, pancreas, thymus gland, suprarenal glands, and mesenteric glands of fatal cases.

The diplococci are Gram-positive and, transplanted to solid media, yield luxuriant growths and a staphylococcus grouping. They grew aerobically and anaerobically, but more slowly under the latter condition, and the cocci became smaller and more rounded. Diplococci were found in the anaerobic cultures of 48 of 106 cerebrospinal fluids; also in the cerebrum, cerebellum, pons and medulla, cord, tonsils, lungs, liver, kidneys, spleen, pancreas, and mesenteric glands of fatal cases.

The filtrates of emulsions of tissues containing streptococci and diplococci passed through fine Kitasato and Pasteur-Chamberland filters were sterile unless large amounts of filtrates were collected. The amount of filtrate collected and cultured is therefore of considerable importance in filtration experiments.

The small forms of streptococci and diplococci in old anaerobic cultures are filterable with these filters, while young aerobic cultures containing large forms are not, unless large amounts of culture are filtered.

Intracranial, intravenous, and intraperitoneal injection of these easily cultivated streptococci, diplococci, diphtheroids, and Gram-negative bacilli failed to produce paralysis in rabbits or monkeys. With two exceptions all the cultures were transplants from the original anaerobic ascites-broth-kidney cultures of cerebrospinal fluid and various tissues. Arthritis and meningitis were produced by the streptococci, but there were neither clinical nor histological evidences of true poliomyelitis.

Occasional bacteriological studies since 1898 have shown that easily cultivated micrococci and bacilli may be present in the cerebrospinal fluid and tissues of the central nervous system of persons suffering with acute anterior poliomyelitis. The majority of bacteriol-

ogists have found the cerebrospinal fluid, blood, and nervous organs sterile. Opinions have varied in regard to the significance of the organisms and the micrococci in particular, but the consensus of opinion has been to the effect that they are secondary invaders and unable of themselves to produce poliomyelitis in the lower animals. After allowing for contaminations due to technical errors in securing specimens, the total number of observations indicates that easily cultivated micrococci occur sometimes in the brain and cord of persons suffering from epidemic poliomyelitis. Our studies have shown that they may be found not only in these locations, but also in the spleen, kidneys, suprarenal glands, and other organs. It is not known that they exert an influence in this disease, although they may possibly give rise to the production of antibodies, assuming their entrance not to be wholly agonal, as the cultures of streptococci are frequently of sufficient virulence to produce meningitis in rabbits and monkeys. Our experiments are in accord with those of other investigators who found that these microorganisms do not produce poliomyelitis in the lower animals, and are therefore in sharp contrast with the recent reports which would attribute an etiologic relationship of streptococci and allied organisms to that disease. At present this wide divergence of result cannot be accounted for, but it does not seem that it is possible for it to reside in any condition of the cultures employed by us as they were obtained from undoubted cases of epidemic poliomyelitis and inoculated in early generations.

As regards these easily cultivatable microorganisms, we agree at present with those who regard them as secondary and probably terminal invaders rather than the actual etiologic agent of the disease.

BIBLIOGRAPHY.

1. Flexner, S., and Noguchi, H., Experiments on the Cultivation of the Microorganism Causing Epidemic Poliomyelitis, *J. Exp. Med.*, 1913, xviii, 461.
2. Mathers, G., Some Bacteriologic Observations on Epidemic Poliomyelitis, *J. Am. Med. Assn.*, 1916, lxvii, 1019.
3. Rosenow, E. C., Towne, E. B., and Wheeler, G. W., The Etiology of Epidemic Poliomyelitis, *J. Am. Med. Assn.*, 1916, lxvii, 1202.
4. Nuzum, J. W., and Herzog, M., Experimental Studies in the Etiology of Acute Epidemic Poliomyelitis, *J. Am. Med. Assn.*, 1916, lxvii, 1205.

5. Bülow-Hansen and Harbitz, F., Bidrag til læren om den akute poliomyelit (Contributions á l'étude de la poliomyélite aigüe. Rés., 1261), *Norsk Mag. Lægevidensk.*, 1898, xiii, 1170.
6. Harbitz, F., and Scheel, O., The Microbe of Poliomyelitis, *J. Am. Med. Assn.*, 1908, l, 281.
7. Harbitz and Scheel, Epidemic Acute Poliomyelitis in Norway in the Years 1903–1906, *J. Am. Med. Assn.*, 1907, xlix, 1420.
8. Geirsvold, M., Epidemisk poliomyelit bakteriologiske undersögeleser, *Norsk Mag. Lægevidensk.*, 1905, iii, 1280.
9. Pasteur, W., Foulerton, A. G. R., and Maccormac, H., On a Case of Acute Poliomyelitis Associated with a Diplococcal Infection of the Spinal Sac, *Arch. Middlesex Hosp. London*, 1908, xii, 13 (also *Lancet*, 1908, i, 484).
10. Leiner, C., and von Wiesner, R., Experimentelle Untersuchungen über Poliomyelitis acuta. IV, *Wien. klin. Woch.*, 1910, xxiii, 817.
11. Krause, P., and Meinicke, E., Zur Aetiologie der akuten epidemischen Kinderlähmung, *Deutsch. med. Woch.*, 1909, xxxv, 1825.
12. Flexner, S., and Lewis, P. A., The Nature of the Virus of Epidemic Poliomyelitis, *J. Am. Med. Assn.*, 1909, liii, 2095.
13. Landsteiner, K., and Levaditi, C., La transmission de la paralysie infantile aux singes, *Compt. rend. Soc. biol.*, 1909, lxvii, 592.
14. Fox, H., *Rep. Comm. Health Penn.*, 1907, 420.
15. Schamberg, J. F., Kolmer, J. A., Ringer, A. I., and Raiziss, G. W., Research Studies in Psoriasis, *J. Cutan. Dis.*, 1913, xxxi, 698.
16. Abramson, H. L., *J. Am. Med. Assn.*, 1917, lxviii, 546.

EXPLANATION OF PLATE 66.

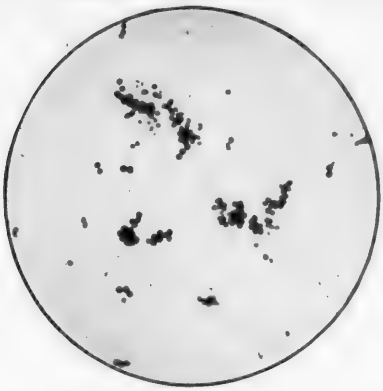
FIG. 1. Film of a 2 day aerobic subculture of diplococcus in ascites-broth-kidney medium; from an anaerobic culture of poliomyelitic cord in the same medium. Shows large forms. × 1,000.

FIG. 2. Film of an 18 day anaerobic culture of diplococcus in ascites-broth-kidney medium; same culture as shown in Fig. 1. The cocci have become smaller in size and more easily decolorized by alcohol in the Gram stain. × 1,000.

FIG. 3. Film of a 5 day anaerobic culture of streptococcus in ascites-broth-kidney medium; from the pons of a fatal case of acute anterior poliomyelitis. × 1,000.

FIG. 4. Film of an 11 day anaerobic culture of streptococcus in ascites-broth-kidney medium; same culture as shown in Fig. 3; some smaller forms of cocci tending to become Gram-negative are seen. × 1,000.

FIG. 5. Film of a 3 day aerobic culture of streptococcus in ascites-broth-kidney medium; from the thymus gland of a fatal case of acute anterior poliomyelitis. × 1,000.



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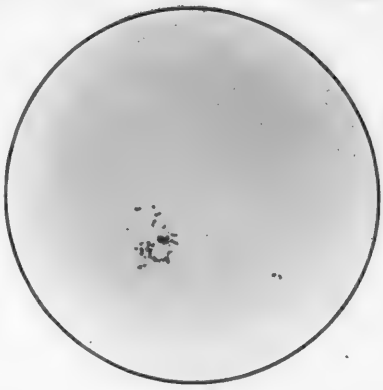

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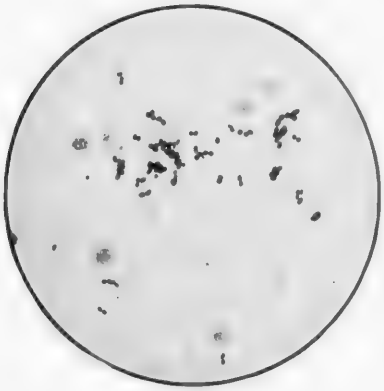

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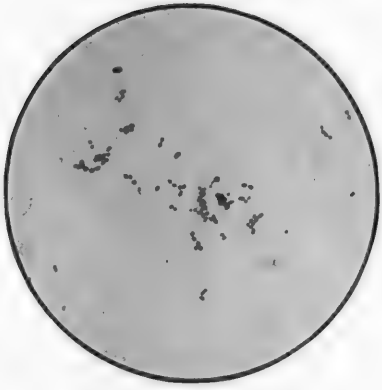

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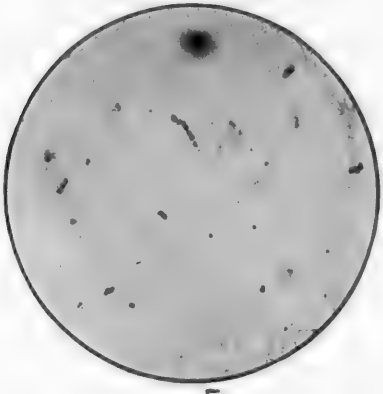


5

(Kolmer: Bacteriology of Poliomyelitis.)

HENLE'S REACTION OF THE CHROMAFFIN CELLS IN THE ADRENALS, AND THE MICROSCOPIC TEST FOR ADRENALIN.

By TOMOSABURO OGATA and AKIRA OGATA.

(*From the Pathological Institute, and the Chemical Laboratory of the Pharmaceutical Institute of the Imperial University of Tokyo, Tokyo, Japan.*)

Plate 67.

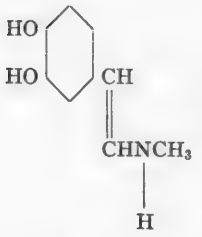
(Received for publication, January 11, 1917.)

HISTORICAL.

In 1865 Henle (1) discovered a brown coloration of the medullary cells of the adrenals when remaining in a solution of potassium dichromate, which at that time was generally used in histology for the hardening of tissues. Henle's reaction has since been confirmed by many investigators, and was also found to occur in other parts of the body; *e.g.*, in the carotid gland, in the sympathetic ganglia, in Zuckerkandl's gland, etc. The cells with a positive reaction are always found in groups, mixed with sympathetic ganglion cells; and numerous embryological investigations have made it clear that both are derived from the same ectodermal mother cells. On the other hand, comparative anatomical studies have shown that the cortex (interrenal system) and the medulla (suprarenal system) of the adrenals are genetically foreign to each other, and in lower vertebrates exist as two independent organic systems. On the basis of these results it can scarcely be doubted that the medullary portion of the adrenals represents, with all tissues giving a positive chrome reaction, a system which stands in close relation to the sympathetic nervous system (Kohn (2), Biedl (3), Wiesel (4, 5)). This characteristic behavior towards chromates was taken into consideration in naming these cells; *e.g.*, chromaffin (Kohn (2)), chromophil (Stilling (6)), chrome brown, or pheochrome (Poll (7)). The term chromaffin is the most commonly used. This term, as Kohn has stated, expresses only the ability of the cells, tissue, or system to be colored brown by dichromate or chromic acid, when in a fresh state, while with the chromaffin substance of Borberg and Ciaccio (17, 18) the term chromaffin indicates chemical affinity. While the chrome reaction was thus established as a characteristic of the chromaffin cells, numerous chemicophysiological investigations convinced us, on the other hand, that these cells produce adrenalin and supply it to the vascular system. This substance is considered an im-

portant hormone for the regulation of the vascular tonus. Langlois and Vincent were the first to show that adrenal extract causes an increase of blood pressure. Later Takamine isolated from adrenal extract a chemical compound which increased blood pressure, and which he named adrenalin. According to some authors (Biedl, Wiesel, etc.), the extract of extracapsular chromaffin tissues, *e.g.*, those of Zuckerkandl's gland, shows the same property of raising blood pressure as that of the adrenals. It is thus clear that adrenalin is a constant chemical constituent of chromaffin tissues. Biedl proposed the terms adrenai cells, adrenal tissue, adrenal bodies, adrenal organs, and adrenal system. He did not, however, always use the term adrenal cells, but substituted for it that of chromaffin cells (Kohn). In France the terms "*cellules adrénalogènes*" (Bonnamour (8)) and "*cellules adrénalinogènes*" have since been in use.

As has been explained above, two constant characteristics, affinity for chromic acid and chromates (Henle's reaction) as a histological, and the production of adrenalin as a physiological characteristic, were established in certain kinds of cells. However, it has not yet been made entirely clear whether these two peculiarities exist accidentally or whether there is a distinct relation between them. While the experiments of Kahn (10), Schur and Wiesel (11), etc., showed parallelism between the degree of the reaction and the adrenalin content, contrary results were published by Ingier and Schmorl (12), Popielski (13), and others. Biedl states that Henle's chrome reaction can serve only as a preliminary orientation as to the quantity of adrenalin contained. Though the presence of a definite relation between the two phenomena is assumed, proof of it is lacking. On the other hand, some investigators, as Mulon (14), Elliott and Tuckett (16), Ciaccio (17), Borberg (18), and others, believe that the chrome reaction is a purely chemical process, which occurs between chromates (chromic acid) and adrenalin or its derivatives. Borberg described this process in detail. He stated that adrenalin solution after the addition of a solution of potassium dichromate gradually becomes more and more reddish brown until in the course of a few hours a slowly increasing dark brown precipitate appears. According to him, the precipitate showed, besides its similarity in color, in its behavior towards various solvents complete agreement with the chrome reaction in the adrenal medulla. He therefore identified both reactions in the test-tube and on the slide. The reaction in the test-tube he explained thus: that potassium dichromate or the chromic acid resulting from the interaction of this salt and the acetic acid in the solution forms an insoluble brownish chemical compound. But he believed that the reaction on the slide was caused not by adrenalin itself but by substances in the preliminary stage of adrenalin formation (adrenalinogen, pre-adrenalin), which would be oxidized into adrenalin derivatives combined with chromates. This so called chromaffin substance may have, according to his hypothesis, the following chemical structure.



Chromaffin substance.

The relation between the chrome reaction and the presence of adrenalin is, however, not yet proved. This is why Biedl maintains that the chrome reaction could only serve as a preliminary orientation as to the quantity of adrenalin contained.

EXPERIMENTAL.

We believe that we have succeeded in solving this question. Our materials were mostly the fresh adrenals of healthy domestic animals, as cattle, horses, pigs, sheep, dogs, rabbits, guinea pigs, and rats. Human adrenals were also used, but were found not to be so well suited for our purpose as in them the reaction was much weaker than in the fresh adrenals of healthy animals. We also investigated in the test-tube the chemical process between adrenalin and potassium chromate, for the reaction can here be easily studied. It was only later we learned that Borberg had also investigated the reaction in this manner. But his results were different from ours. We too observed that an adrenalin solution turns brown on the addition of a solution of potassium chromate, to be followed by a dark brown precipitate which slowly increases. At first we believed with him that the precipitate was a chemical compound of chromic acid with some derivative of adrenalin, which arises by oxidation from adrenalin and dissolves in water, showing red coloration, for we perceived, in mixing both solutions, first the change of color into red and then the brownish precipitate. By closer examination of the properties of the brownish precipitate we learned that the latter was not a complicated organic compound but a simple inorganic one, namely chromium dioxide (CrO_2).

There are various methods for producing chromium dioxide; for instance, by introducing nitric oxide into a solution of potassium chro-

mate (Schweitzer (19)), by adding a solution of potassium chromate to a solution of chromium sulfate (Maus (20)), by digesting chromic acid solution and chromium hydroxide (Beusch (21)), by adding a solution of sodium thiosulfate to one of potassium chromate (Popp (22)), or reducing potassium dichromate by sunlight in the presence of alcohol (Vogel (23)). The process may be explained thus: that chromates are first reduced to chromium dioxide and the latter combines with the existing chromic acid into chromium dioxide according to the following equation.

$$K_2Cr_2O_7 + 4H_2O - 3O = Cr_2(OH)_6 + 2KOH$$
$$Cr_2(OH)_6 + K_2Cr_2O_7 = CrO_3.Cr_2O_3 + 3H_2O + K_2CrO_4$$

It is conceivable that adrenalin, which is easily oxidized and therefore can exert a reducing effect upon other compounds, *e.g.*, the reduction compounds mentioned above, extracts part of the oxygen from the potassium dichromate, thus forming chromium dioxide.

If in the experiment mentioned above the precipitate in the test-tube is examined, it is found to agree in appearance and other properties with chromium dioxide, which has been extracted according to one of the methods described. The precipitate is a brownish powder and appears under the microscope in the form of yellow-brown amorphous granules, is insoluble in various solvents, such as alcohol, ether, chloroform, xylol, benzene, etc., but is easily soluble in caustic potash and mineral acids. In acetic acid and formic acid it is not easily soluble, but if these organic acids are present in the original mixture its elimination is interfered with. When dried at 100°C. it becomes dark brown; when brought to a glow it gives off no carbon dioxide, proving the absence of organic compounds. Quick heating causes it to flare up, as Schweitzer has observed with chromium dioxide.

If this precipitate, which we believe to be chromium dioxide, is washed, the water appears yellow on account of the admixture of a surplus of potassium dichromate, and further washings make the color disappear to a yellowish trace which cannot be got rid of. The same fact has been observed with chromium dioxide. Schiff (24) washed 1 gm. of chromium dioxide for 10 hours daily for 6 weeks, but chromic acid was still seen to dissolve. Popp also mentions that if chromium dioxide is washed until color no longer appears, it changes

to a new compound, $2Cr_2O_3.CrO_3$. For explanation of this chromium dioxide might be regarded as a loose compound of chromium oxide and chromic acid, so that in washing, a part of the chromic acid is continually dissolved. When the brownish precipitate was allowed to remain for several days on the water bath, a greenish shade of color gradually appeared; and if dried at a gradually increasing temperature it became black and gradually decreased in weight. This agrees with the fact observed with chromium dioxide, that its hydroxide does not easily give off water, and only under high temperature changes into green chromium oxide, setting oxygen free.

In order to convince ourselves further that the precipitate is chromium dioxide, we made it glow and examined the residue (chromium oxide). The method was as follows. To a 1 per cent solution of adrenalin hydrochloride a solution of potassium dichromate was added in excess; it was left for 3 days at 37°C., centrifuged, and poured off. To this, water was added and the same operation was repeated several times, until the upper layer retained the same yellowish color. Absolute alcohol was then added to the precipitate, the mixture was shaken for 30 minutes, again centrifuged, and treated with ether, after which a brown powder was obtained, which was dried at 170°C. in a platinum crucible until the weight remained constant. This powder was gradually heated to red heat and the green residue, chromium dioxide, was weighed.

0.2026 gm. substance gave 0.1648 gm. chromium oxide.

0.1648 gm. of chromium oxide gave 0.1822 gm. of $2CrO_2$ or 0.2017 gm. of $2CrO_2 + H_2O$.

From this it follows that the substance must have the molecular formula $2CrO_2 + H_2O$.

The following formulas have been given for the hydrate of chromium dioxide, dried at various temperatures.

Observer.	Formula.	Temperature.
		°C.
Popp.	$2Cr_2O_3.CrO_3 + 9H_2O$	Not given.
Kopp.	$CrO_2 + 2H_2O$	Room.
Vogel.	$CrO_2 + H_2O$	100
Hinz.	$CrO_2 + \frac{1}{2}H_2O$	200
"	CrO_2	250

As the substance was dried at 170°C., the formula $2CrO_2 + H_2O$ ($CrO_2 + \frac{1}{2}H_2O$) stands almost exactly between the third and fourth, and also agrees in this respect with the supposed hydrate of chromium dioxide.

As chromium dioxide is an amorphous substance, only a relative purity can be reached by washing. If the latter is continued too long, the chromium dioxide molecule loses part of its chromic acid; if it is not continued long enough, the precipitate may still contain some potassium chromate. It is therefore necessary to follow exactly the method of others in order to compare the results.

By the above investigation we believe we have established that the precipitate obtained by mixing solutions of adrenalin and dichromate in the test-tube is chromium dioxide.

We then prepared frozen sections of the adrenals, in which we had effected a chrome reaction. We left them in the solutions for a long time to test the conditions of solubility. In these sections we were able to ascertain the same conditions as those in the test-tubes. The shade of color in the sections was exactly that of chromium dioxide. By heating chromated adrenals to red heat we obtained a relatively large amount of chromium oxide. Though we were unable to analyze directly the brownish substance in the tissues, we may assume, according to the above investigation, that Henle's reaction is simply a reducing phenomenon of adrenalin upon dichromate of chromic acid. If the amount of adrenalin is great, a correspondingly large amount of chromium dioxide will be formed by reduction, so that the estimation of the content of adrenalin from the degree of the chrome reaction has thus been proved to be also theoretically correct. As the chrome reaction in the test-tube has shown, it is most distinct in a neutral medium; an acid or alkali medium disturbs the reaction. This explains why the chrome reaction in dead bodies is not proportional to the content of adrenalin, on account of postmortem changes which also affect the chemical reaction of tissues.

The above considerations explain why the chrome reaction in sections is most distinct in simple solutions of potassium dichromate. It can also be obtained with Mueller's fluid, Orth's fluid, dichromate-formalin solution, etc., because the other admixtures to these solutions do not disturb the reaction of the dichromate. In Zenker's

fluid, however, the chrome reaction becomes indistinct, not only because its constituent sublimate disturbs the reaction, but also because the large addition of acetic acid prevents the elimination of chromium dioxide.

As Henle has noted, the reaction can also be produced by chromic acid (Merck), but according to our observation, in the test-tube as well as in the sections, chromic acid must in this case be used only in a greatly diluted solution; 0.3 to 0.5 per cent is best. The reason is that the extracted chromium dioxide dissolves again in the abundant chromic acid. But microscopically the reaction caused by chromic acid is somewhat different on the slide. With chromic acid the atrabiliary part of the adrenals is of a diffuse brownish color, while with dichromate brownish granules appear in the protoplasm of the chromaffin cells. This may possibly be explained by the fact that the eliminated chromium dioxide is dissolved by the abundance of chromic acid, causing the diffuse coloration.

We have tried the reaction also with a solution of ammonium dichromate. The result was the same as with potassium dichromate.

The chrome reaction may also be effected by chromates as well as by dichromate and chromic acid. With chromates (we used potassium chromate), however, a large amount of adrenalin seems to be necessary for the reduction, for with the 1 per cent commercial adrenalin solution we could not produce the reaction in the test-tube, or in human materials, but it appeared distinctly in the fresh adrenals of healthy animals (rat, dog, pig). This reaction is similar to that of chromic acid,—microscopically it shows diffuse coloration.

We found that the chrome reaction appears best in the sections with 5 per cent solution of potassium dichromate, without any admixtures. But with this there is not sufficient hardening of the tissue, so we put the tissue first into 5 per cent potassium chromate solution for 6 to 12 hours, then added a tenth part of formalin, which gave the necessary hardening.

We also tried to combine the chrome reaction with subsequent coloring which some investigators have recommended for making the reaction more distinct. Such methods are Dewitzky's (25) (cresyl purple coloring) and Wiesel's (water blue safranine or toluidine blue safranine double coloring), but the simple toluidine blue coloring

recommended by Borberg (18) gave better results (discoloring in alcohol). We found that the grade of reaction can be estimated best without subsequent staining.

Since we have thus established that Henle's reaction is a simple reduction phenomenon of adrenalin upon chromic acid, especially dichromate, it follows that this phenomenon should not only occur with chromium compounds but also with other inorganic compounds. We have looked for such compounds, which should be easily reducible and insoluble in water. We first tried whether the reaction occurs in the test-tube, and if we obtained a precipitate we employed the medium also on tissues. But every reaction which succeeded in the test-tube was not always applicable to tissues. For instance, in a solution of potassium permanganate, manganese is at once precipitated by the addition of adrenalin, but because in tissues other organic substances besides adrenalin will reduce potassium permanganate, the adrenalin reaction is not recognizable by itself.

Fehling's solution, too, is reduced in the test-tube by adrenalin, but gave negative results in tissues. Fehling's solution contains a comparatively large amount of caustic soda, in consequence of which the adrenalin passes from the tissue into the solution and no copper oxide precipitates in the tissue.

We could easily reduce osmic acid by adrenalin in the test-tube. By using it on the adrenals the medulla becomes black but after this the lipoids of the cortex are also more markedly black. Afterwards we found that Mulon (15) in 1905 had observed the same phenomenon and also explained it as a reaction by adrenalin. He investigated the lipoid of the adrenals and came upon this fact by accident.

We made experiments with nitrate of silver. A solution of it is easily reduced by adrenalin in the test-tube and silver is precipitated; in the tissue of the adrenal medulla also precipitation takes place, but some precipitation occurs in all parts of the tissue, not only in the medulla; moreover, the simple solution does not penetrate deep into the tissue. We have, therefore, made various experiments and shall describe our method.

After completing this investigation we found that a former investigator had already observed this silver reaction of the adrenals and likewise explained it as a reduction by adrenalin. Laignel-Lavastine (26, 27), in the course of extensive

histological investigations of the sympathetic nerves, also examined the nerve filaments of the adrenal medulla. He applied Kajal's silver impregnation on the adrenals of a rabbit and accidentally found a silver precipitate in the medullary cells. He believed that adrenalin was the cause of this phenomenon.

We also tried Kajal's method, but as we had found already with the simple nitrate of silver solution, silver is precipitated throughout the whole tissue and the method is therefore not applicable for the microscopic reaction of adrenalin.

In our own method we employed ammoniacal silver solution as follows: (1) Cut the adrenals (chromaffin tissue) with a sharp knife into sections as thin as possible; (2) place the sections without previous fixing in 1 per cent ammonium solution for 2 hours; (3) transfer to 5 per cent ammoniacal silver solution for 3 to 5 hours; (4) place again in 1 per cent ammonium solution, repeating this several times, for 30 minutes; (5) transfer to 3 per cent sodium thiosulfate solution for 1 hour (these operations, Nos. 3 to 5, must be made in the dark room, protected from daylight); (6) wash in running water for 1 hour; (7) fix in 10 per cent formalin solution; (8) prepare sections by any method.

If the reaction is positive the medullary cells are filled with black and silver granules. Silver granules were also found in blood vessels, which also occurs with the chrome reaction and indicates the presence of adrenalin in the blood vessels.

Da Costa (28) reported his application of Wilshowsky's silver method in the adrenals. According to an abstract, he obtained results similar to ours, but as we have not seen the original paper we do not know his method of procedure. Wilshowsky's method refers only to tissues fixed in formalin. A reduction by adrenalin cannot take place. We believe, therefore, that our silver method is different from Da Costa's.

SUMMARY.

We have established the fact that the chrome reaction as well as the silver and osmium reactions are merely reductions by adrenalin. In our opinion the naming of the cells giving a positive reaction should not be based upon the reaction (*i.e.*, chromaffin cells), but on the presence of adrenalin itself. Biedl's terms, adrenal cell, adrenal organ,

adrenal body, adrenal system, and also Bonnamour's term, adrenalin-producing cells, are appropriate in this respect. We propose the names adrenalin cell, adrenalin tissue, adrenalin system, thereby indicating the presence of adrenalin.

BIBLIOGRAPHY.

1. Henle, J., Über das Gewebe der Nebenniere und der Hypophyse, *Z. rationelle Med.*, 1865, xxiv, 142.
2. Kohn, A., Die Paraganglien, *Arch. mikr. Anat.*, 1903, lxii, 263.
3. Biedl, A., Innere Sekretion, ihre physiologischen Grundlagen und ihre Bedeutung für die Pathologie, Vienna, 2nd edition, 1913.
4. Wiesel, J., Zur Pathologie des chromaffinen Systemes, *Virchows Arch. path. Anat.*, 1904, clxxvi, 103.
5. Wiesel, Krankheiten der Nebennieren, in Lewandowsky, M., Handbuch der Neurologie, Berlin, 1913, iv, 348.
6. Stilling, H., Zur Anatomie der Nebennieren, *Arch. mikr. Anat.*, 1898, lii, 176.
7. Poll, H., Die vergleichende Entwicklungsgeschichte der Nebennierensysteme der Wirbeltiere, *Handb. vergl. u. exp. Entwicklungsgesch.*, 1906, iii, 442.
8. Bonnamour, S., Recherches histologiques sur la sécrétion des capsules surrénales, *Compt. rend. Assoc. anat., Montpellier*, 1902, iv, 54.
9. Bonnamour, Étude histologique des phénomènes de sécrétion de la capsule surrénale chez les mammifères, Lyon, 1905.
10. Kahn, R. H., Zuckerstich und Nebennieren, *Arch. ges. Physiol.*, 1911, cxl, 209.
11. Schur, H., and Wiesel, J., Beiträge zur Physiologie und Pathologie des chromaffinen Gewebes, *Verhandl. deutsch. path. Gesellsch.*, 1907, xi, 175; *Wien. klin. Woch.*, 1907, xx, 1202.
12. Ingier, A., and Schmorl, G., Über den Adrenalingehalt der Nebennieren, *Deutsch. Arch. klin. Med.*, 1911, civ, 125.
13. Popielski, L., O wewnetrznem wydzielanin nadnerczy (Internal Secretion of the Suprarenal Glands), *Lwow. tygodn. lek.*, 1912, vii, 341.
14. Mulon, P., Spécificité de la réaction chromaffine: glandes adrénalogènes, *Compt. rend. Soc. biol.*, 1904, lvi, 113.
15. Mulon, Sur la réaction osmique de la médullaire des surrénales, *Compt. rend. Soc. biol.*, 1905, lvii, 757.
16. Elliott, T. R., and Tuckett, I., Cortex and Medulla in the Suprarenal Glands, *J. Physiol.*, 1906, xxxiv, 332.
17. Ciaccio, C., Sur la sécrétion de la couche médullaire de la surrénale, *Compt. rend. Soc. biol.*, 1906, lx, 332.
18. Borberg, N. C., Das chromaffine Gewebe, *Skand. Arch. Physiol.*, 1913, xxviii, 91.
19. Schweitzer, E., Über die Doppelsalze des chromsauren Kali's mit der chromsauren Talkerde, *J. prakt. Chem.*, 1846, xxxix, 257.

20. Maus, *Poggendorfs Annalen*, ix.
21. Beusch, *Poggendorfs Annalen*, lv.
22. Popp, O., Ueber das chromsaure Chromoxyd, *Ann. Chem. u. Pharm.*, 1870, clvi, 90.
23. Vogel, A., Jr., Über chromsaures Chromoxyd, *J. prakt. Chem.*, 1859, lxxvii, 482.
24. Schiff, H., Ueber Chromsuperoxyd und Chromsäure, *Ann. Chem. u. Pharm.*, 1861, cxx, 207; Über Chromsuperoxyd, 1874, clxxi, 116.
25. Dewitzky, W., Beiträge zur Histologie der Nebennieren, *Beitr. path. Anat. u. allg. Path.*, 1912, lii, 431.
26. Laignel-Lavastine, Application de l'imprégnation argentique de Cajal à l'étude histo-chimique de la cellule médullo-surrénale, *Compt. rend. Soc. biol.*, 1905, lviii, 661.
27. Laignel-Lavastine, Note sur la médullaire des surrénales normales du lapin, *Bull. Soc. anat.*, 1905, lxxx, 331.
28. da Costa, XV Congr. Int. Med., Sect. I, Anat., Lisbon, 1906, 393.

EXPLANATION OF PLATE 67.

Silver impregnation according to Ogata's method.

FIG. 1. Adrenal of pig.

FIG. 2. Human adrenal (beri-beri).

The chromaffin cells in the medulla of the adrenals are strongly impregnated.

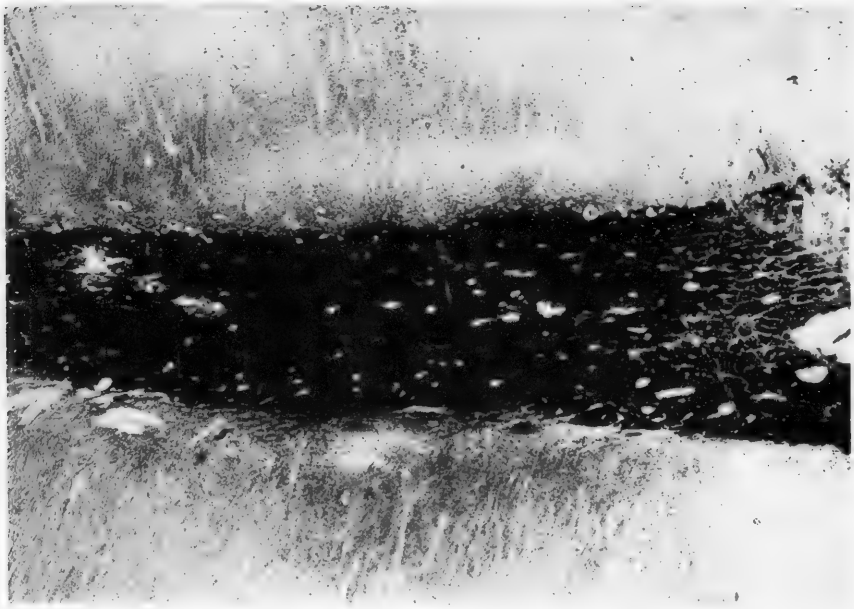


FIG. 1.

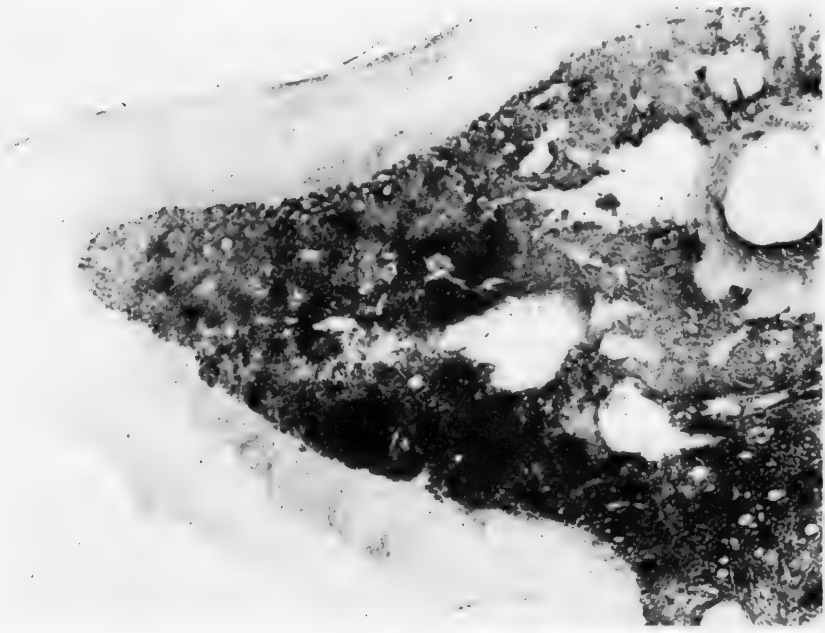


FIG. 2.

(Ogata and Ogata: Henle's Reaction of Chromaffin Cells.)

STUDIES ON BACILLUS WELCHII WITH SPECIAL REFERENCE TO GAS GANGRENE.*

By J. P. SIMONDS, M.D.

(From the Department of Pathology, of Northwestern University Medical School, Chicago, and the Research Laboratory, Ambulance de l'Océan, La Panne, Belgium.)

(Received for publication, December 11, 1916.)

Gas gangrene is preeminently an infection associated with war wounds. This is perhaps not due to any great difference in the degree of contamination of wounds received on the battle-field as compared with the injuries resulting from accidents in civil life. Spores of the group of bacteria capable of causing gas gangrene are probably no more abundant in the mud of the trenches than in ordinary street dirt, for they are known to have a wide distribution. The marked difference in the frequency of the disease in times of war and of peace is probably to be attributed to the dissimilarity in the character of the wounds encountered. In ordinary accidents, injuries associated with laceration and befouling of the tissues are usually too wide open to afford favorable conditions of growth to anaerobic bacteria which alone are able to cause typical gas gangrene. Shrapnel balls and, especially, fragments of highly explosive shells, on the other hand, produce penetrating wounds characterized by a comparatively narrow canal with ragged walls composed of lacerated tissues. Bits of clothing and other débris are frequently carried into the wound. The narrow channel becomes more or less completely filled with blood clot, and, thus sealed in, anaerobic bacteria are able to grow.

It must be remembered in this connection that these organisms enter the wounds in the form of spores. It is not improbable that spores of anaerobic bacteria require more favorable conditions for germination than vigorously growing vegetative forms demand for their continued multiplication. It is just these favorable conditions of an-

* Aided by a grant from The Rockefeller Institute for Medical Research.

aerobiosis and suitable culture medium that are furnished in many war wounds.

In spite of the study of many sporadic cases of gas gangrene in civil practice, much remains to be learned concerning the pathogenesis of this infection. It was with the hope of discovering something as to the conditions essential to the occurrence of this disease in contaminated wounds that these studies were undertaken. As a preliminary investigation, the uniforms of a number of Belgian soldiers were examined to determine what percentage of them carried spores of this group of bacteria. Material—foreign bodies, lacerated tissues, etc.—removed from fresh wounds was then studied to find what proportion of these contained bacteria of this group. At the same time an effort was made to ascertain in what part of the wound germination of the spores was most prompt and complete. These patients were observed and the number that later developed gas gangrene was noted. It was also found convenient to study *in vitro* certain therapeutic measures employed in the treatment of wounds in general and of gas bacillus infections in particular.

The Examination of the Uniforms of Belgian Soldiers for Spores of Anaerobic Bacteria Capable of Causing Gas Gangrene.

The importance of the contamination of war wounds with bits of clothing in the causation of gaseous gangrene has been well recognized. Except for a few unpublished preliminary experiments by Dr. Alexis Carrel, I know of no systematic study of the uniforms of soldiers with reference to the presence of spores of bacteria capable of causing this disease. The examinations here reported were made in the Research Laboratory of the Ambulance de l'Océan at La Panne, Belgium. Unfortunately, on account of the difficulty of obtaining laboratory supplies in the War Zone, especially in a position so near the trenches as La Panne, it was impossible to test the virulence and pathogenicity of the organisms found or to do more than make the simple tests for group identification. The results of the work are presented, however, with the belief that they have some practical value.

These investigations were undertaken with three definite objects in view: (1) to determine the relative frequency with which the uniforms

carry spores of bacteria of the group known to cause gaseous gangrene; (2) to determine the efficiency of the sterilization of uniforms as practiced in the Belgian Army; and (3) to determine the time in which the uniforms become reinfected after the sterilization.

The materials studied consisted of the following: (1) The uniforms of twelve soldiers who had been in La Panne for 8 days to 3 weeks and away from the trenches for a slightly longer period. During this time their uniforms had been previously sterilized twice in four instances and once in each of the other eight. (2) The uniforms of thirty soldiers directly from the trenches. These were examined before and immediately after sterilization, and on each of the 3 succeeding days. During this time the men remained in La Panne. (3) Twenty new uniforms from the factory at Gravelines. (4) Samples of the cloth from which the uniforms were made.

The technique employed in the examination of the uniforms was simple. An area of approximately 5 sq. cm. on the outer surface of the blouse was selected, on the left side between the upper and lower pockets, and on the front of the trousers just above the knee. This area was washed several times with about 10 cc. of sterile water contained in a sterile Petri dish, over the edge of which the area of uniform was held. The water was drawn up several times in a sterile glass tube about 20 cm. long, and was allowed to flow repeatedly over the area under examination, the surface being rubbed at the same time with the end of the tube. 2 cc. of these washings were inoculated into tubes of whole milk recently sterilized in the autoclave. The tubes were then heated at 80°C. for 15 minutes to kill all vegetative forms of bacteria. The recent sterilization rendered the milk anaerobic, and the further heating at 80°C. expelled the air dissolved in the water used for inoculation. A sufficient degree of anaerobiosis was thus easily obtained for the growth of organisms of the group of *Bacillus welchii*.

The examinations before and immediately after sterilization of the uniforms were made at the Military Baths in La Panne,[1] under exactly similar conditions. This furnished a satisfactory control on

[1] For this privilege and for many courtesies we are indebted to Capt. Rolland, Officer in Charge of the Military Baths of the Belgian Army.

the method used. The results presented in the tables below indicate that there was no contamination due to faulty technique.

In examining the samples of cloth two methods were used: (1) The surface of the fabric was rubbed with a sterile swab moistened with sterile water. The swab was then placed in a tube of sterile anaerobic milk and heated to 80°C. for 15 minutes. (2) Small squares of the same size were cut from the cloth and passed through the free flame until the surface was thoroughly singed, or, in many cases, even charred. A series of control tests made by gently rubbing the burned surfaces of these squares with a moist sterile swab and inoculating broth and milk showed that the destruction of bacteria on the surface had been complete. The few cases in which these control tests showed that the surface sterilization was not perfect are not included in the tables given below. On scraping away the burned outer portion or on cutting across the square of cloth, the interior part of the goods was found almost unchanged. The singed or charred squares were placed in tubes of anaerobic milk and incubated either with or without previously heating to 80°C. for 15 minutes. Samples of cloth known to contain spores of *Bacillus welchii* were wrapped in gauze and placed in the pockets of uniforms ready to be sterilized at the Military Baths. After sterilization squares of these samples inoculated into anaerobic milk yielded no growth.

In all instances stained films from the milk cultures were examined. Inasmuch as the medium employed was especially favorable to the growth of the gas bacillus, very few other microorganisms were encountered in these studies. Those found were spore-forming bacteria which either (1) grew in cultures inoculated with washings which did not contain *Bacillus welchii*, or (2) were able to multiply in an acid medium and therefore grew in association with the latter organism.

The results of these examinations are shown in Tables I to IV.

It is seen from Table I that in this group 100 per cent of the trousers carried spores of anaerobic bacteria, and that more than 80 per cent contained spores of *Bacillus welchii*. The blouses were a little less frequently infected than the trousers. In the case of three of these garments no anaerobic bacteria grew in the cultures. Sterilization was thorough.

TABLE I.

Examination of the Uniforms of Twelve Soldiers Who Had Been in La Panne on Coast Guard Duty for 8 to 20 Days.

	Blouses.	Trousers.
Total number showing spores of anaerobic bacteria before sterilization	9	12
Total number showing spores of *B. welchii* before sterilization	9	10
Total number showing spores of bacteria of any kind after sterilization	0	0

TABLE II.

Examination of Uniforms of Thirty Soldiers from the Trenches.

Time of examination.	Garment.	Number with spores of anaerobic bacteria.	
		All kinds.	Group *B. welchii*.
Before sterilization	Blouses.	30	27
	Trousers.	30	27
Immediately after sterilization	Blouses.	0	0
	Trousers.	2	2
24 hrs. " "	Blouses.	7	7
	Trousers.	15	13
48 " " "	Blouses.	10	9
	Trousers.	20	20
3 days " "	Blouses.	14	11
	Trousers.	18	16

In Table II the first column of figures shows the number of garments containing spores of anaerobic bacteria of all kinds and therefore includes the figures in the second column. In this group of thirty uniforms, 100 per cent carried spores of anaerobic bacteria and 90 per cent had the spores of *Bacillus welchii*. The sterilization appeared not to be so effective in the case of these heavily contaminated garments as in the case of the previous group.

After the sterilization of their uniforms the soldiers remained in La Panne. The time of reappearance of the spores of *Bacillus welchii* upon these uniforms is also shown in Table II. It is evident that the spores of these bacteria were much less numerous on the uniforms 3

days after sterilization than when the men arrived from the trenches. It is on account of the comparatively slight recontamination that a garment which yielded a positive result on one day sometimes gave a negative one on the following day. With so few spores present it was possible to miss them. This is the explanation of the fact that, as shown in Table II, a larger number of trousers showed spores of *Bacillus welchii* 48 hours after sterilization than 72 hours after.

In many instances during the 3 days succeeding sterilization, 48 to 72 hours were required for the development of the typical reaction in milk inoculated with washings from the uniforms. Before sterilization this reaction developed in from 18 to 24 hours. This is indirect evidence of the comparative paucity of spores of the gas bacillus during the 3 days on which the examinations were made.

TABLE III.

Examination of Twenty New (Unused) Uniforms.

Number of cases in which spores of anaerobic bacteria were present	Blouses.	9
	Trousers.	9
Number of cases in which spores of *B. welchii* were present	Blouses.	7
	Trousers.	9

It is seen from Table III that a notable percentage of the new (unused) uniforms carry spores of *Bacillus welchii*. Our results show less than 50 per cent so contaminated. It is probable, however, that these figures are too low. In the first place, they are much lower than the percentage of positive results obtained from an examination of samples of the cloth from which the uniforms were made (Table IV). Furthermore, the cloth of the new uniforms was so oily that it was impossible to bring the water into close contact with the goods. Any spores which may have been present, therefore, were dislodged with difficulty.

The samples of woolen cloth used in the experiments summarized in Table IV were obtained from the factory at Gravelines, and were identical with the cloth of the uniforms of the Belgian Army. The samples of cotton goods, few in number, came from the same source and represented the material used in making pockets and linings of uniforms. From this table it is evident that 100 per cent of the sam-

TABLE IV.

Examinations of Samples of Cloth from Which the Uniforms Were Made. Total Number of Samples Examined: Wool, 48; Cotton, 10.

Sample of cloth.	*B. welchii.*	Not sterilized.	Flamed.	Sterilized at baths.
Wool.....	Present.	23	14	0
	Absent.	0	3	8
Cotton.....	Present.	5	0	0
	Absent.	0	1	4

ples carried spores of *Bacillus welchii*. In the woolen goods the spores were in the meshes of the cloth. In the three instances in which negative results followed flaming of the sample, the process was prolonged until almost the whole mass was charred.

Spores of this organism are common in the gastro-intestinal tract of sheep and are constantly present in the soil. They were, therefore, undoubtedly on the wool when it was clipped from the animals. Whether these spores were not completely removed from the wool in the process of manufacture, or whether others were carried into the thread or woven cloth by the solutions used in dying or waterproofing, cannot now be stated.

It is evident from the above facts (1) that the new uniforms as received by the soldiers of the Belgian Army and the cloth from which these garments are made contain spores of *Bacillus welchii* in the meshes of the goods; (2) that 100 per cent of the uniforms of men from the trenches carry spores of anaerobic bacteria, and of these, 90 per cent carry the spores of the *Bacillus welchii* group; (3) that after sterilization of the uniforms, about 50 per cent of the trousers became contaminated with spores of the *Bacillus welchii* group during the first 24 hours, and about 66.66 per cent in the first 3 days when the men remain in barracks.

The Presence of Bacillus welchii in Recent War Wounds in Relation to the Subsequent Development of Gas Gangrene.

In the bacteriologic study of the uniforms of soldiers directly from the trenches, it was found that about 90 per cent carried spores of organisms morphologically like *Bacillus welchii* capable of causing

stormy fermentation of milk. The frequency with which bits of clothing are carried into wounds by fragments of shell and shrapnel balls has often been commented upon. It seemed desirable, therefore, to determine in what percentage of cases bacteria of this group could be isolated from fresh wounds, and to follow these cases in order to discover what proportion of them later developed gaseous gangrene.

Through the courtesy and cooperation of Dr. Depage, Surgical Director of the Ambulance de l'Océan at La Panne, it was possible to obtain material from twenty-one such cases. One of the patients, débris from whose wounds yielded *Bacillus welchii*, died of shock about 2 hours after reaching the Ambulance. The remaining twenty cases will therefore be made the basis of this report.

The material received for examination was removed by the surgeon under aseptic precautions and placed in sterile Petri dishes soon after the patient entered the hospital and, usually, within 2 to 8 hours after the reception of the wound. It consisted of bits of clothing and other débris, fragments of shell, lacerated tissues, and blood clots. Tubes of sterile anaerobic milk were inoculated, in duplicate in most instances, with pieces of the material. Duplicate cultures were made from muscle and from connective tissue (and fat) when these were removed from the wound. Since these bacteria were carried into the wounds in the form of spores, it was important to determine whether these spores had vegetated, and in what part of the wound and in what tissues this most readily occurred. One set of tubes thus inoculated was, therefore, heated to 80°C. for 15 minutes and then incubated. The other set of tubes was placed in the incubator without heating. In many cases the bacilli were isolated in pure culture and accurately identified.

Of the 20 cases, 5 proved entirely negative. Of these, only one contained pieces of clothing. In this case, unfortunately, only a heated culture was made. Of these 5 negative cases, none developed gaseous gangrene.

In the fifteen remaining cases, bacteria resembling *Bacillus welchii* were present. In ten of these, pieces of clothing were found in the wound; in the other five only fragments of shell and damaged tissues were removed.

Of the fifteen cases, three developed gaseous gangrene within the

first 48 hours after entering the hospital. The first of these was a young soldier with multiple shell wounds of the legs, on the service of Dr. Anten and Dr. Lagasse. He developed a mild localized gaseous gangrene around a wound about the middle of the right thigh. Under treatment, recovery was prompt. The other two cases were on the service of Dr. Van de Velde. In one of these a bullet entered the right thigh, shattered the femur, passed through the scrotum, and lodged in the muscles of the left thigh. The third case was a compound fracture of the right femur by a fragment of a hand grenade. Both of these patients developed severe gaseous gangrene of the right thigh, which was, however, controlled without resorting to amputation.

Stained films of the discharge from the wounds, in all twenty cases, were examined every 2 days. In the three cases which developed gaseous gangrene, microorganisms morphologically resembling *Bacillus welchii* were present in abundance at the beginning, but disappeared promptly after the subsidence of the gangrene. In two other of the fifteen positive cases a few bacilli similar to *Bacillus welchii* were seen in the first films examined, but were not present on subsequent examinations. In the remainder of the fifteen cases none of the films showed any bacilli that could be called *Bacillus welchii*.

In regard to the part of the wound in which vegetation of the spores is most prompt and complete, the two cases mentioned above on the service of Dr. Van de Velde yielded notable results. Pieces of damaged muscle and of connective tissue and fat were received from both cases in such a condition that separate cultures could easily be made. In both instances the heated and unheated milk tests were positive in the case of connective tissue and fat. In the case of the damaged muscle, however, the heated milk tubes remained sterile while the unheated duplicate tubes gave almost pure cultures of *Bacillus welchii*. Both of these patients developed gaseous gangrene. This same difference between injured muscle and connective tissue was also evident in other cases. From this, one would seem to be justified in concluding that the spores which are carried into the damaged muscle vegetate quickly.

This observation is important theoretically because it indicates the probable mode of origin of gaseous gangrene, and practically because

it suggests the prophylactic measures to be employed for its prevention. The spores which only reach the more superficial connective tissue are more exposed to the air and therefore find it difficult, if not impossible, to vegetate. In the more deeply lying muscle, on the other hand, a more perfect condition of anaerobiosis is attained. Furthermore, the exudate in the injured connective tissue is composed of lymph, blood, and blood serum; that is, a protein medium containing little or no sugar. In the lacerated muscle, on the contrary, one finds, in addition to the above exudate, the glycogen of the torn muscle fibers. Here, then, are the conditions favorable for the growth of *Bacillus welchii;* namely, anaerobiosis and a medium fairly rich in fermentable carbohydrate. It is in the injured muscle, therefore, that the spores first germinate and in which the gas, which is a most important factor in the spread of the bacilli and the advance of the infection in the tissues, is produced in abundance.

Bacillus welchii is not motile and cannot of itself change its position in the exudate of the infected area. It is a large heavy bacillus and is not readily transported through the narrow tissue spaces by the sluggish lymph. This organism produces substances which appear to be negatively chemotactic; and it is exceptional to find a bacillus in any of the few leukocytes present in the exudate in the wound. In the dissemination of the bacilli in the tissues adjacent to the wound it is the gas produced by them, the pressure of which may be very high,[2] that appears to be the most important factor. By forcing their way into the loose subcutaneous, inter- and intramuscular connective tissue, the bubbles of gas carry the bacilli with them, by the combined action of capillary attraction and surface tension, more and more deeply into the surrounding healthy tissues.

The onset and progress of gaseous gangrene may be visualized somewhat as follows. Bacteria capable of causing this disease are carried into the wound in the form of spores. Those which lodge in the more superficial connective tissue find conditions unfavorable to growth both as to the degree of anaerobiosis and as to the character of the culture medium. Those which are carried into the more deeply placed lacerated muscle find conditions perfectly adapted to their

[2] Taylor, K., *Lancet*, 1916, i, 123.

needs and therefore germinate and multiply rapidly. Gas is produced in abundance from the glycogen of the crushed and torn muscle fibers. The bacilli are forced along the loose connective tissue by the infiltrating gas among the more healthy muscle bundles, and the infection advances. The rapidity of its progress depends in part, at least, upon the amount and pressure of the gas and the looseness of the connective tissue.

It is difficult, and usually impossible, to obtain growth of *Bacillus welchii* in pure protein media such as sugar-free broth or sometimes even in plain broth, unless large quantities of an actively fermenting culture are used for inoculation. This is especially true if other bacteria are present. In such a favorable medium as milk or dextrose agar, on the other hand, there is evidence that very small numbers of vegetative forms or spores of this microorganism, perhaps even single spores,[3] will multiply rapidly, and easily outgrow other bacteria present. An almost equally favorable medium is found in lacerated muscle tissue. One may therefore conclude that when the spores have once been carried into the wound, the most important element in their germination and the onset of the infection is the damaged muscle with its anaerobiosis and fermentable carbohydrate. When the spores have vegetated and vigorous growth begins, the important elements in the spread of the infection are the pressure of the gas and the loose connective tissue. The further rapid growth of *Bacillus weichii* in the wound may be due either (1) to the heavy inoculation of the more purely protein exudate of the connective tissue from the growth in the muscle; or (2) to the further liberation of glycogen from other muscle fibers killed by the action of the acid products of fermentation or by pressure of the gas. That the latter is more probable is supported by the histologic studies of Taylor.[4]

In the various attempts to explain the pathogenesis of gaseous gangrene much emphasis has been laid upon the fact that nearly all cases of this infection occur in the extremities,[5] especially the lower. These

[3] Simonds, J. P., Monograph of The Rockefeller Institute for Medical Research, No. 5, 1915, 34, 85.

[4] Taylor, *J. Path. and Bacteriol.*, 1915–16, xx, 384.

[5] For a full bibliography, with brief abstracts, of papers which have appeared in French and English journals, see Moiroud, P., and Vignes, H., La gangrene gazeuse, et les plaies gangreneuses, Paris, 1916.

are the locations in which large masses of muscle are found. Of the cases in this series which showed *Bacillus welchii* in the fresh wounds but failed to develop gaseous gangrene, none were wounds involving injury to large masses of muscle. They were either superficial injuries, or wounds of the head, face, trunk, joints, or feet where muscle tissue is not very abundant.

Summarizing these results, it was shown that in this series of twenty cases, 75 per cent of the fresh wounds contained spores or vegetative forms of bacilli morphologically resembling *Bacillus welchii* and capable of producing stormy fermentation of milk. In most cases the organisms were isolated in pure culture and identified. Only 20 per cent of this number, *i.e.*, 15 per cent of the whole series, developed gaseous gangrene. It is only in the injured muscle that these spores find conditions favorable to immediate germination and rapid multiplication; namely, anaerobiosis and a culture medium rich in fermentable carbohydrate. In the pathogenesis of this infection in contaminated wounds, therefore, the lacerated muscle is the most important factor, and it is to this that attention should be directed in the prevention of the disease.

The Influence of Different Concentrations of Saccharose upon the Growth of Bacillus welchii.

It is well known from the work of Theobald Smith[6] and of Kendall[7] and his coworkers that the presence of fermentable carbohydrate in culture media favors the growth and profoundly modifies the metabolism of many bacteria. Concentrated solutions of sugars, on the contrary, either kill most bacteria or inhibit their growth. It was probably these two principles which led to the use of sugar in the treatment of war wounds. In the case of wounds infected with ordinary pathogenic bacteria this method of treatment may have a reasonable theoretical basis. In the case of wounds contaminated with spores of *Bacillus welchii*, however, one would have good reason to fear that the application of sugar might bring disastrous results.

[6] Smith, T., *Tr. Assn. Am. Phys.*, 1896, xi, 37.

[7] Kendall, A. I., *J. Biol. Chem.*, 1912–15. Kendall, A. I., and Walker, A. W., *Tr. Chicago Path. Soc.*, 1915, ix, 320.

This microorganism can be made to grow only with difficulty in sugar-free media. In media containing almost any carbohydrate, however, growth is surprisingly rapid,—at times almost explosive in character. It is probable that none of this group of bacteria produce a soluble toxin; certainly not in the presence of fermentable sugar. This group is distinctly not putrefactive. It is purely fermentative. It is from sugar that they produce irritating and injurious substances in the form of butyric and other organic acids.[8] It has been noted that in a series of fresh wounds it was found that 75 per cent contained either the spores or the vegetative forms of *Bacillus welchii*. For this reason it seemed desirable to study experimentally the effect of different concentrations of sugar upon the multiplication and gas production of this bacillus.

Ten strains of *Bacillus welchii* isolated either from distinct cases of gaseous gangrene or from débris found in fresh wounds were selected for study. Bouillon containing 5, 10, 20, 40, 50, and 60 per cent respectively of saccharose was sterilized in the autoclave in fermentation tubes and in ordinary test-tubes under oil, cooled, and inoculated.

Three methods of inoculation were employed: (1) tubes of all concentrations were inoculated from a 24 hour milk culture; (2) subcultures in the 5 per cent saccharose broth were made from a milk culture, incubated for 24 hours, and inoculations were made from these into the higher concentrations; (3) transplants were made from a 24 hour culture in each dilution into the next higher; that is, from 5 per cent into 10 per cent, from 10 per cent into 20 per cent, and so on through the series. The results were the same with all three methods. During the period covered by these experiments there was no visible tendency for any of the strains gradually to become accustomed to growth in the presence of high concentrations of sugar. In a few instances there was a small amount of gas in tubes of 50 per cent saccharose inoculated from a 24 hour milk culture, while no gas was found in a duplicate 50 per cent tube inoculated from a 24 hour culture of the same strain in 40 per cent sugar broth.

These experiments were repeated a number of times with different lots of media but with the same concentrations of sugar. Characteris-

[8] Herter, C. A., The Common Bacteriological Infections of the Digestive Tract and the Intoxications Arising from Them, New York, 1907.

tic results are shown in Table V in which the amount of gas produced in the closed arm of a fermentation tube has been measured in the usual manner. Since the inoculations were always made from one fluid culture into another of higher specific gravity, the organisms carried over in inoculating rose immediately to the top of the closed arm of the fermentation tube where the condition of anaerobiosis was ideal.

TABLE V.

Culture.	Saccharose.					
	5 per cent.	10 per cent.	20 per cent.	40 per cent.	50 per cent.	60 per cent.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
617 c	70	60	80	40	0	0
669 b	70	90	90	60	5	0
285	80	65	90	50	60	0
365 a*	85	70	90	85	Trace.	0
365 b*	85	70	90	60	0	0
793 a*	70	80	75	80	0	0
793 b*	—	80	—	40	60	0
797 a	70	100	80	75	10	0
"Central"	40	95	95	70	Trace.	0
386 cd	70	60	55	85	0	0

* 365 a and 365 b were fished from separate colonies in the original cultures from the same case. The same is true of 793 a and 793 b.

From this table it is evident that *Bacillus welchii* can produce an abundance of gas in broth containing amounts of saccharose up to 400 gm. per liter. Some strains are able to ferment this sugar in 50 per cent concentration. Of the ten strains studied, not one was able to produce gas in 60 per cent saccharose broth. The bacteria in these cultures were not always dead, for in many instances anaerobic milk, inoculated from 60 per cent saccharose broth tubes that had been in the incubator for 3 days, showed stormy fermentation. It was observed that in the dilutions up to 20 per cent, fermentation was very rapid and was practically complete in 24 hours. Production of gas proceeded much more slowly in the higher concentrations. At the end of 24 hours there was only a small bubble of gas in the 40 per cent tubes and in the 50 per cent tubes which showed any gas production. The process required 48 to 72 hours for completion. In a few instances

there appeared to be some growth of the bacilli in 50 per cent saccharose broth, as evidenced by the increased turbidity in the upper part of the closed arm of the fermentation tube, without the production of gas.

Stained films were made after 24 to 48 hours and examined to determine the effect upon morphology of placing these bacilli in solutions of such high specific gravity as the 50 and 60 per cent saccharose solutions. Except for a possible slight increase in thickness and a tendency to stain palely, there were no detectable morphologic differences between bacilli grown in the higher concentrations of the sugar and those grown in the weaker concentrations.

Because the presence of fermentable sugar is essential to the vigorous growth of *Bacillus welchii*, and because saccharose has been used in the treatment of war wounds, at least 75 per cent of which are contaminated with spores of this organism, it seemed desirable to study the effects of different concentrations of sugar upon its growth. All the strains studied grew and produced an abundance of gas in broth containing 5, 10, 20, and 40 per cent respectively of saccharose. In the 40 per cent solution formation of gas was somewhat slower than in the lower concentrations. Only two strains produced gas in abundance, somewhat slowly, in 50 per cent saccharose broth. None of the strains grew or produced gas in broth containing 60 per cent saccharose.

The Effect of Pure Oxygen Gas on Cultures of Bacillus welchii.

Injections of pure oxygen gas directly into the tissues is used as a routine treatment for gas gangrene in many of the military hospitals in France and Belgium.[9] In a previous study[10] of *Bacillus welchii* it was observed that the degree of anaerobiosis in milk cultures influenced materially the type of reaction of these organisms in this medium. Variations were noted from no growth in milk saturated with

[9] Vennin, Girode, and Haller, *Bull. et mêm. Soc. chir., Paris,* March 9, 1915; for further references and abstracts see Moiroud and Vignes, La gangrene gazeuse, Paris, 1916.

[10] Simonds, Monograph of The Rockefeller Institute for Medical Research, No. 5, 1915, 36.

air, to coagulation with little or no gas formation in tubes containing small amounts of air, and to typical stormy fermentation in milk with the optimum degree of anaerobiosis. It seemed possible, therefore, that the efficacy of the injections of oxygen might not depend solely on their supposed bactericidal action. A study of the effect of pure oxygen gas upon cultures of *Bacillus welchii* was undertaken.

The following technique was employed. Tubes of anaerobic milk or dextrose broth under oil were inoculated with *Bacillus welchii.* Oxygen was passed into the medium by means of a capillary pipette which reached to the bottom of the tube. The gas rose through the liquid in a series of many minute bubbles. After oxygenation the tubes were placed in the incubator, and at intervals subcultures were made in fresh anaerobic milk or deep dextrose agar by transferring about 0.5 cc. of the culture into the new medium. In another series of experiments tubes of milk or dextrose broth were inoculated with *Bacillus welchii,* incubated for 3 or 4 hours, and then oxygenated for 20 minutes as described above. Typical results from numerous experiments are collected in Table VI.

It is evident from Table VI that strains of *Bacillus welchii* differ in their resistance to oxygen. Strains 617 a and 617 ds are much more readily killed by oxygen than Strain 365 a. Whether the resistance is in inverse proportion to the virulence of the organism cannot be stated. But Strain 365 a was isolated from a mild localized case of gas gangrene, and when its pathogenicity for guinea pigs was tested for the first time about 3 months after its isolation, it was found to be wholly non-virulent. Strain 617 a, on the other hand, was obtained from a severe case of gas gangrene, and after 3 months' growth on artificial media caused the death of a guinea pig in 24 hours.

When tubes of milk inoculated with *Bacillus welchii* were incubated for 3 hours before the oxygen gas was bubbled through them, the oxygenation did not appear to inhibit their growth in any way. Even Strain 617 a, unincubated cultures of which were sterile in less than 2 hours after passage of the gas for 20 minutes, was not affected by 20 minutes' oxygenation if the culture had been previously incubated for 3 hours.

In instances in which the organisms were not actually killed by the oxygen it will be noted that there was a marked reduction in the

TABLE VI.

Culture.	Time in incubator before oxygenation.	Time of passage of oxygen gas.	Original culture after incubation.			Subcultures made after			
			24 hrs.	48 hrs.	72 hrs.	2 hrs.	4 hrs.	6 hrs.	24 hrs.
	hrs.	*min.*							
365 a	0	5	++++			++++		++++	
365 a	0	10	+++			++++		+++	
365 a	0	20	+++			++++		+++	
365 a	3	20	++++						
386 cb	0	5	+++			++		+	++++
386 cb	0	10	−	−	+	++		+	+
386 cb	0	20	−	−	+	++		+	+
386 cb	3	20	++++						
617 ds*	0	5	+++			++		++	
617 ds	0	10	−	−	−	+		−	−
617 ds	0	20	−	−	−	+		−	−
617 ds	3	20	++++						
617 a*	0	10	−	−	−	−	−	−	
617 c*	0	10	+++			+++		+++	
617 c	0	15	−	−	−	+	+	−	−
553 a	0	5	−	+	+	++	+		+
553 a	0	10	−	−	−	++	−	−	−

++++ indicates typical stormy fermentation; +++, coagulation with less gas than typical stormy fermentation; ++, coagulation with small amount of gas; +, coagulation with no gas; −, no change in medium.

* Cultures 617 a and 617 c are two strains isolated from the same case of gas gangrene. Strain 617 ds is Strain 617 a after it had been passed through a guinea pig in which it produced a fatal gas gangrene.

production of gas in the culture. This may have been due in part to the restraining effect of the oxygen on the multiplication of the bacilli so that there were too few organisms present to produce the usual amount of gas. But this same character of depressed gas formation was carried on into subsequent generations. Films from all these cultures showed evidence of considerable growth, although it was not possible to estimate accurately the relative amount in the different tubes. It seems, therefore, that exposure to oxygen may depress the biologic activity of gas bacilli without actually causing their death. This lowered activity can be carried on through a number of generations even when grown in as favorable a medium as milk. It is

not associated, however, with the acquirement of any new or strange characters such as ready spore formation or liquefaction of gelatin. These depressed cultures can be easily rejuvenated by growth for a generation or two in anaerobic dextrose-liver-broth medium.

It might be mentioned that in studying organisms of this group, no biologic reactions should be carried out except with recently rejuvenated cultures. Only in this way can uniform and characteristic results be obtained.

From the experiments described above one may suggest as practical possibilities (1) that injections of oxygen gas will be much more effective if made early in the course of gas gangrene than if delayed until the growth of the infecting organisms has acquired considerable energy and momentum; and (2) that the good effects of the injection of oxygen may not be due wholly to its bactericidal effects but partly to its power to depress the vital activity of *Bacillus welchii* whereby less gas is produced.

SUMMARY.

1. Spores of the *Bacillus welchii* group of bacteria were found on 100 per cent of the uniforms of Belgian soldiers who had come directly from the trenches, and in the meshes of all the samples examined of the new cloth from which the uniforms were made.

2. In fifteen out of twenty fresh war wounds members of this group of bacteria were found. Of the fifteen patients, only three later developed gas gangrene. Once the spores of *Bacillus welchii* have been carried into a wound the deep-lying lacerated muscle tissue appears to be the most important factor in the onset of gas gangrene.

3. *Bacillus welchii* is able to grow and produce gas in broth containing up to 40 per cent saccharose. Some strains were able to multiply and produce gas in 50 per cent saccharose broth; but none of those examined were able to grow when the concentration of the sugar reached 60 per cent.

4. The bubbling of pure oxygen through milk or dextrose broth cultures of *Bacillus welchii* has a definite depressor action on the production of gas. This does not appear to be due to a reduced number of organisms in the culture.

EQUILIBRIA IN PRECIPITIN REACTIONS.

The Coexistence of a Single Free Antigen and Its Antibody in the Same Serum.*

By STANHOPE BAYNE-JONES, M.D.

(From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.)

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The vagueness of the terms of serology connotes the difficulties with which the describers of this new knowledge have had to contend. Unable to use simple and definite chemical substances, and without the guidance of any general theory, they have had to employ mass adjectives to describe the reactions of a serum as a whole, and have gathered great numbers of observations without any plan for their coordination. Whenever, therefore, it is possible to repeat former experiments by substituting known factors in place of unknown mixtures, the work should be undertaken. Quantitative data will be obtained through these studies which will eventually allow serology to find a place in either chemistry or physics, or what seems almost self-evident, in physical chemistry. The colloidal state of matter has afforded so many analogies to the reactions of serology that the terms of colloid chemistry have already been applied with uncritical facility by immunologists to reactions which have not yet been studied with quantitative accuracy sufficient to indicate their true nature. The systematic attempt, however, to prove or disprove these colloidal analogies in serology will undoubtedly result in a unifying conception of the principles of the reactions of immunity.

The precipitation of protein by specific sera has many analogies with colloidal reactions and this phenomenon lends itself readily to investigation simplified by the use of at least a single pure substance.

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Zinsser[1] has emphasized the colloidal phases of the precipitin reaction, and recently Weil[2] has repeated the experiments, using crystallized egg albumin as the antigen. One of the phases of the precipitin reaction which has engaged particular attention is that in which the precipitinogen and the precipitin occur simultaneously but ununited in the same serum. This is easily demonstrable when whole serum or a mixture of proteins is used as the antigen. Weil, however, using crystalline egg albumin as the antigen was unable to demonstrate the coexistence of free antigen and antibody in the same serum. It is of considerable theoretical importance to confirm this finding. The results obtained by Weil are so little in accord with expectations based upon chemical or colloidal theories that it was considered advisable to repeat the experiments on precipitin reactions with crystalline egg albumin. The following studies, therefore, were carried out under the direction of Dr. Zinsser, with some modifications of recent work on this reaction.

HISTORICAL.

In 1902 Linossier and Lemoine,[3] Ascoli,[4] and Eisenberg[5] noticed that when foreign serum had been injected into rabbits in large doses the sera of these animals contained both antigen and antibody. They used whole sera as antigens. To demonstrate the simultaneous presence of free precipitable substance and its precipitant in the same serum, fractions of the serum were tested, first for antigen, by adding another antiserum, and second for precipitin by adding an homologous antigen. Gay and Rusk[6] have described the same phenomena, and by showing that the sera do not fix complement, although they contain antigen and antibody, they have furnished additional evidence of the ununited state of the substances.

Zinsser and Young[7] have pointed out that the association of free antigen and its antibody in serum, as shown by the failure of this serum to fix complement, is evidence that the reaction does not take place according to the law of mass

[1] Zinsser, H., Infection and Resistance, London and New York, 1914, 266.

[2] Weil, R., *J. Immunol.*, 1916, i, 19.

[3] Linossier, G., and Lemoine, G.-H., *Compt. rend. Soc. biol.*, 1902, liv, 85.

[4] Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 1409.

[5] Eisenberg, P., *Centr. Bakteriol., Ite Abt., Orig.*, 1903, xxxi, 773.

[6] Gay, F. P., and Rusk, G. Y., *Univ. California Publications, Pathology*, 1912, ii, 59, 73.

[7] Zinsser, H., and Young. S. W., *J. Exp. Med.*, 1913, xvii, 396.

action. According to the formula of that law, this serum should not only contain free antigen and antibody, but also a certain quantity of the united complex. To explain the inhibition of the union of antigen and antibody in such sera Zinsser and Young[7] have drawn a strict parallel with the protective colloidal reactions. These experiments show that it is not improbable that the serum proteins act as protective colloids, preventing the precipitation of a protein by its antiserum, just as gum arabic will protect colloidal arsenic sulfide against the precipitating action of gelatin.

Von Dungern[8] confirmed the findings of Linossier and Lemoine but explained them according to a different hypothesis. He showed that whole serum consists of a number of protein complexes, all of which cause the formation of precipitin in the immunized animal. One portion of the protein mixture may have more antigenic properties than another, as a result of which partial precipitins of various strengths are produced for the whole serum. Von Dungern believed that these complex antigens and multiple antibodies reacted irregularly in such a way that while the supernatant fluid of a precipitin test would contain free antigens found in part in the original serum together with precipitins capable of flocculating the original serum, yet the antisubstances in the supernatant fluid were not strictly homologous and hence would not react with each other. His extensive experiments have had great influence upon subsequent opinions. He worked, however, entirely with multiple antigens and gave only inferences and analogies for the precipitin reactions with pure proteins.

At first glance it would seem as if the experiments in the precipitation of casein with lactoserum, reported by Müller,[9] were evidence that with purified antigens the phenomenon of Linossier and Lemoine did not occur. The supernatant fluids after his precipitin reactions never contained free antigen along with antibody. His experiments, however, are not relevant to the results of precipitin reaction with purified protein. Müller used whole milk as his casein, obviously a multiple antigen, and the degrees of dilutions used in the tests leave such large gaps that the zone in which antigen and antibody coexist ununited might have been overlooked.

Weil[2] confirms all the previous observations on the coexistence of free antigen and antibody in the supernatant fluids of precipitin tests when the whole serum, raw egg white, or any mixed protein is used. With purified egg albumin as antigen, the results were different. He states: "If this antigen is mixed in graded proportions with the serum of a rabbit immunized thereto, and the resulting precipitates are removed by centrifugation, the supernatant fluid never contains both antigen and antibody; either one is present alone." The protocols of Weil's precipitin tests allow him to draw sharp conclusions between the effect of 0.005 cc. and 0.004 cc. of the antigen, a delicacy of reaction which not all workers have

[8] von Dungern, F., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 355.

[9] Müller, P. T., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 48.

been able to attain. The crystalline egg albumin, prepared for him by Coca, was certainly as pure as it is possible to obtain this substance. It is not stated whether anaphylactic reactions were carried out with this particular preparation to determine whether it was thereby free from antigen complexes which cause reactions in animals sensitized with egg globulin. Weil,[2] however, referring to his earlier work with Coca[10] states that he "found that if egg albumin and egg globulin were separated by chemical means, and different rabbits were immunized to each substance, the resulting immune sera reacted powerfully each with its own antigen, and very weakly with the other antigen." This anaphylactic interrelation of egg proteins will be discussed later. It is significant here to note the apparent discrepancy between the results of these precipitin tests and anaphylactic reactions, although one antigen was used which admittedly gives some anaphylactic reaction with other proteins.

EXPERIMENTAL.

Sera capable of precipitating egg albumin were prepared by immunizing rabbits with intravenous injections of this protein. By varying the proportions of immune serum and solution of egg albumin in the precipitin tests, zones of dilution were obtained in which tests for free antigen and free antibody could be made. In addition, by testing the sera of immunized rabbits at certain intervals after the injection of albumin, the proportions and duration of circulating antigen and antibody could be studied.

The important material for these experiments is the antigen. Attempts to use casein failed because of the confusing precipitates caused by the calcium salts required in the tests with this protein. In some experiments, pure edestin from hemp-seed was used.[11] The results with this protein were definite. Because, however, of the slight solubility of edestin, work with it is somewhat difficult. Crystalline egg albumin was found to be most suitable for these studies. This substance was prepared[12] according to the method of Hopkins and Pinkus.[13] The whites of freshly laid eggs were carefully precipitated with ammonium sulfate and the albumin from these was

[10] Weil, R., and Coca, A. F., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 141.

[11] The preparation was made by Dr. Harris of the Connecticut Agricultural Experiment Station.

[12] The substance was prepared under the direction of Mr. Balls of the Department of Physiological Chemistry of Columbia University.

[13] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

recrystallized three times. The solution of protein was dialyzed under toluene in the ice box to free it as much as possible from ammonium sulfate and acetic acid. The albumin was then dried *in vacuo* to a white glistening powder. The Zsigmondy "gold number"[14] for the preparation was 8, showing the usual protective value of pure egg albumin as a colloid. This preparation met the requirements for pure crystalline egg albumin and was probably as pure as it is possible to obtain a protein of this nature.

Anaphylactic tests were made in the hope of showing that this preparation of egg albumin was free from globulin or substances which had antigenic properties like globulin. Egg globulin was prepared from the first fraction of egg white precipitated by half saturation with ammonium sulfate. A solution of the precipitate was dissolved in distilled water and dialyzed against distilled water until the globulin began to flocculate. This material was centrifuged and the precipitate taken up in 10 per cent sodium chloride. This formed a turbid emulsion, free, however, from large particles. One series of guinea pigs weighing 250 gm. was sensitized by intravenous injections of varying amounts of crystalline egg albumin; another series by intravenous injections of egg globulin. After an interval of 26 days the animals were reinjected with protein (Table I).

The results of the experiments summarized in Table I show that preparations of egg albumin and egg globulin have a common antigenic factor, capable of producing mutual anaphylactic reactions. They confirm the statements of Wells[15] and Wells and Osborne,[16] who, after a long series of experiments concluded that: "In spite of the most careful separation of these two portions of egg white by means of ammonium sulfate precipitation, the resulting preparations each react almost as well against the other as against itself." Weil and Coca[10] report a somewhat similar experience with crystalline egg albumin when injected into animals sensitized to egg globulin. These reactions have been considered in detail in order to determine whether or not the anaphylactic test is a valid criterion of the purity of a pro-

[14] Schulz, F. N., and Zsigmondy, R., *Beitr. chem. Phys. u. Path.*, 1903, iii, 137.
[15] Wells, H. G., *J. Infect. Dis.*, 1911, ix, 147.
[16] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1913, xii, 341.

TABLE I.

*Anaphylactic Reactions with Solutions of Ovalbumin and Ovoglobulin from the Same Eggs. Guinea Pigs, Weight 200 to 250 Gm., Injected Intravenously on July 13, 1916. Second Injection on August 7, after an Interval of 26 Days.**

Guinea pig No.	Sensitizing dose.	Interval.	Second injection.	Result.
	Animals sensitized to crystalline ovalbumin.			
		days		
1	0.002 gm. of albumin.	26	0.1 gm. of albumin.	Convulsions. Died.
2	0.002 " " "	26	0.2 " " "	" " in 2 hrs.
3	0.001 " " "	26	0.1 " " "	Typical anaphylaxis. Died in 3 min.
4	0.001 " " "	26	0.1 " " "	Convulsions. Died in 3 min.
5	0.0005 " " "	26	0.1 " " "	Violent convulsions. Died in 2 min.
6	0.002 gm. of albumin.	26	0.1 gm. of globulin.	Convulsions. Died in 3 min.
7	0.002 " " "	26	0.1 " " "	Died in 30 min.
8	0.001 " " "	26	0.002 " " "	Diarrhea. Survived.
9	0.001 " " "	26	0.002 " " "	Sneezing, coughing. Survived.
	Animals sensitized to ovoglobulin.			
10	0.004 gm. of globulin.	26	0.1 gm. of globulin.	Convulsions. Died in 1 min.
11	0.002 " " "	26	0.002 " " "	" " " "
12	0.002 " " "	26	0.002 " " "	" Paralysis in hind legs. Died in 2 min.
13	0.001 " " "	26	0.002 " " "	Cough. Survived.
14	0.001 " " "	26	0.002 " " "	Convulsions. Died in 2 min.
15	0.0005 " " "	26	0.002 " " "	" " " "
16	0.002 gm. of globulin.	26	0.1 gm. of albumin.	Cough. Survived.
17	0.002 " " "	26	0.1 " " "	Sneezing, dyspnea. Survived.
18	0.001 " " "	26	0.1 " " "	Sprawling, twitching. Survived.
19	0.001 " " "	26	0.1 " " "	Cough. Survived.
20	0.0005 " " "	26	0.1 " " "	" "

* Separate syringes and needles were used for each protein.

tein, and in particular whether the anaphylactic interreactions of egg globulin and egg albumin exclude the latter from use as a single pure antigen. Crystalline egg albumin is acknowledged to be a pure protein.[15,17] Egg globulin, however, probably consists of ovomucin and ovalbumin admixed in indefinite proportions.[15] The impurity of the globulin renders the anaphylactic reactions without significance. In the protocols of Table I, it is seen that the albumin-globulin reactions are much weaker than those caused by the injection of the specific protein into a sensitized animal. This undoubt-

EXPERIMENT I.

Edestin as Antigen.

This experiment was done to show coexistence of edestin and its precipitin in the serum of an immunized rabbit.

Apr. 15–18, 1916. Serum from Rabbit 21, immunized to edestin, obtained 1 hour after an intravenous injection of 12 cc. of 0.027 per cent solution of edestin. This serum was kept for 3 days in the ice box before being tested for its content of antigen and antibody. Serum from Rabbit 22 also immunized to edestin, having a precipitin titer of 1:4,000. These sera were mixed in various proportions. The diluent was a mixture containing 0.9 per cent sodium chloride and 0.105 per cent sodium carbonate in order to keep the edestin in solution.

Tube.	Serum 21.		Edestin.	Chloride-carbonate solution.	Serum 22.		Precipitate.		
	Dilution.	Amount.			Dilution.	Amount.	Ring.	After 1 hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*	*cc.*		*cc.*			
For precipitin.									
1	Undiluted.	0.2	0.2	0.5			±	+	+
2	1 : 5	0.2	0.2	0.5			o	++	++
For antigen.									
3	Undiluted.	0.2		0.5	Undiluted	0.2	+	++	++
4	1 : 100	0.2		0.5	"	0.2	++	+	+
5	1 : 1,000	0.2		0.5	"	0.2	±	±	±
Controls.									
6	Undiluted.			0.7			o	o	o
7				0.7	"	0.2	o	o	o
8			0.2	0.7			o	o	o
9			0.2	0.5	1:4,000	0.2	±	+	+

[17] Schryver, S. B., General Characters of the Proteins, New York, 1909, 20.

edly indicates that the globulin preparation contained ovalbumin. Belief in the individuality of the crystalline egg albumin as an antigen rests upon its chemical characteristics.

Accepting, therefore, purified edestin and ovalbumin as single antigens, the experiments were conducted upon their specific precipitin reactions.

EXPERIMENT II.

Edestin as Antigen.

Apr. 20, 1916. Various quantities of edestin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in supernatant fluids after the primary precipitin reaction.

Serum from Rabbit 22, immunized to edestin, having a titer of 1:2,000.

0.02 per cent solution of edestin mixed in various proportions with undiluted Serum 22.

Tube.	Serum 22, undiluted.	0.02 per cent solution of edestin.	Chloride-carbonate mixture.	Precipitate after 24 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.3 cc. of Serum 22.	0.2 cc. of supernatant fluid plus 0.3 cc. of edestin solution.
	cc.	*cc.*	*cc.*			
1	0.2	2		++	++++	0
2	0.2	1		++	++	±
3	0.2	0.5	0.3	+++	++	±
4	0.2	0.2	0.6	+++	+	+
5	0.2	0.1	0.7	++	Tr.	+
6	0.2	0.01	0.8	+	?	+
7	0.2	0.001	0.8	+	0	++
8	0.2	0.0001	0.8	±	0	++
9	0.2	0.00001	0.8	0	0	++
Controls.						
10	0.2		0.3	0	0	++
11		0.2	0.3	0	++	0

This experiment demonstrates the phase in the precipitin reaction in which a single antigen occurs simultaneously but ununited with its homologous antibody. In the table, the shaded columns overlap each other through the zone in which edestin and antiedestin were found free in the same fluid.

EXPERIMENT III.

Crystalline Egg Albumin as Antigen.

June 15–16, 1916. Various quantities of crystalline ovalbumin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in the supernatant fluids after the primary precipitin reaction. Serum from Rabbit 23 immunized to crystalline egg albumin, having a titer of 1:10,000. 5 per cent crystalline egg albumin in normal salt solution was added in diminishing amounts to undiluted Serum 23 according to the following table.

Tube.	Serum 23, undiluted.	5 per cent crystalline egg albumin.	Normal salt solution.	Precipitate after 18 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.2 cc. of Serum 23.	0.2 cc. of supernatant fluid plus 0.2 cc. of crystalline albumin, diluted 1:10.
	cc.	*cc.*	*cc.*			
1	0.5	0.05	0.25	++	+++	+
2	0.5	0.01	0.25	+++	++	+
3	0.5	0.005	0.25	+++	++	++
4	0.5	0.001	0.25	++++	+	++
5	0.5	0.00075	0.25	+++	±	++
6	0.5	0.0005	0.25	++	o	++
7	0.5	0.0001	0.25	+	o	+++
8	0.5	0.00005	0.25	±	o	+++
Controls.						
9	0.5		0.25	o	o	++++
10		0.5	0.25	o	+	o
11		0.5	0.25	o	+	o

The overlapping shaded areas in the above table show the zone in the supernatant fluids in which free antigen and antibody (crystalline egg albumin and its precipitin) definitely coexist.

EXPERIMENT IV.

Crystalline Egg Albumin as Antigen.

This experiment was done to show coexistence of antigen and antibody in the circulation of an immune animal.

June 2, 1916. Serum from Rabbit 23, immunized to crystalline egg albumin, having a titer of 1:5,000 before the last injection of antigen. 10.45 a.m. 3 cc. of 5 per cent solution of crystalline egg albumin were injected intravenously into Rabbit 23. 11.30 a.m. Bled from carotid 15 cc. 3 p.m. Tests were carried out as follows.

Tube.	Serum 23A before injection.	Serum 23B after injection.	5 per cent solution of crystalline egg albumin.		Normal salt solution.	Normal rabbit serum.	Precipitate after ½ hr. at 37°C.
			Dilution.	Amount.			
	cc.	*cc.*		*cc.*	*cc.*	*cc.*	
For antigen in Serum 23 B.							
1	0.2	0.2					++++
For precipitin in Serum 23 B.							
2		0.2	Undiluted.	0.2			+++
3		0.2	1 : 10	0.2			++
4		0.2	1 : 100	0.2			o
Controls.							
5					0.2		o
6						0.2	o
7	0.2	0.2			0.2		o
8	0.2	0.2				0.2	o
9			Undiluted.	0.2	0.2		o
10			"	0.2		0.2	o
11					0.2	0.2	o

Tube 1 shows that the serum of Rabbit 23 contained antigen ununited with its antibody 45 minutes after an injection of crystalline egg albumin. Tubes 2, 3, and 4 show that during this time free precipitin was still present in the blood of the immune animal.

In several experiments conducted like this one, similar results were obtained, showing that the sera of rabbits immunized to egg albumin contain uncombined antigen and antibody in the circulation for approximately 48 hours after the last injection of the antigen.

In many of the precipitin reactions with crystalline egg albumin the maximum of precipitation occurred only when the albumin solution was diluted from 500 to 1,000 times. This phase of the precipitin reaction is commonly known as the prozone, and is most readily explained as a phenomenon of colloidal relationship. In this particular case it seemed desirable to investigate the inhibition of precipitation effected by the albumin preparation, to relate it, if possible, to the protective influence exerted by this emulsoid on a sensitive gold sol. To show this effect, Experiment V was performed.

EXPERIMENT V.

Protective Action of Egg Albumin.

June 1, 1916. This experiment was done to show the protective action of a solution of crystalline egg albumin upon the precipitation of human serum by its specific antiserum. To the serum from Rabbit 24, having a titer of 1:10,000 against human serum, various quantities of a 5 per cent solution of crystalline egg albumin were mixed with human serum. After ½ hour, anti-human serum from Rabbit 24 was added to these mixtures, as follows. The gold number of this preparation of egg albumin was 8.

Tube.	Human serum.		Anti-human Serum 24.		5 per cent solution of crystalline egg albumin.	Normal salt solution.	Precipitate after ½ hr. at 37°C.
	Dilution.	Amount.	Dilution.	Amount.			
		cc.		*cc.*	*cc.*	*cc.*	
1	Undiluted.	0.2	Undiluted.	0.2		0.2	++++
2	"	0.2	1:5	0.2		0.2	+++
3	1:100	0.2	1:5	0.2		0.2	++
4	1:1,000	0.2	1:5	0.2		0.2	+
5	1:5,000	0.2	1:5	0.2		0.2	+
6	1:10,000	0.2	1:5	0.2		0.2	0
7	Undiluted.	0.2		0.2	0.2		++++
8	"	0.2	1:5	0.2	0.2		++
9	1:100	0.2	1:5	0.2	0.2		++
10	1:100	0.1	1:5	0.1	0.2	0.2	±
11	1:100	0.1	1:5	0.1	0.4		0
Controls.							
12	Undiluted.	0.2				0.2	0
13	"	0.2			0.2		0
14				0.2	0.2		0
15				0.2		0.2	0

These tests (Tubes 7 to 11) show that the presence of sufficient egg albumin prevents the flocculation of human serum by its antiserum. This effect of the solution of egg albumin is in accord with its well known protective action on other colloids, particularly its protection of a sensitive gold sol. against precipitation by electrolytes. The protective action in the above tests is obviously stronger than that exerted by the sera and may explain the long prozones commonly found in precipitin reactions with solutions of crystalline egg albumin.

The hypothesis of von Dungern[8] is insufficient to explain the fact that a serum containing both antigen and antibody, though clear at first, undergoes spontaneous precipitation upon long standing.[1] As

this precipitation progresses the amount of antigen and antibody gradually diminishes. This phenomenon is a matter of common observation when the sera of animals immunized to a mixture of protein are studied at successive intervals after an injection of the proteins used as antigen. Since it was shown by Experiments I and IV that a simple antigen such as edestin or crystalline egg albumin remains ununited with its homologous precipitin in the circulation of an immune rabbit for at least 24 hours after the last injection of the protein, a serum was readily obtained in which the slow spontaneous union of a single antigen with its antibody could be observed. This series of tests was carried out as follows.

EXPERIMENT VI.

Spontaneous Precipitation in a Serum Containing Antigen and Antibody (Crystalline Egg Albumin and Its Precipitin).

July 27, 1916. Rabbit 25 immunized to crystalline egg albumin, having a serum with a titer of 1:25,000, was injected intravenously with 3 cc. of a 5 per cent solution of crystalline egg albumin. 1 hour later blood was drawn aseptically into sterile tubes. Serum obtained from this was divided into several lots and stored in sterile tubes in the ice box. Titrations for antigen and antibody were made as follows.

Tube.	Serum 25 after injection.	Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Normal salt solution.	Normal rabbit serum.	Precipitate.	
			Dilution.	Amount.			After ½ hr. at 37°C.	After 24 hrs. in ice box.
	cc.	*cc.*		*cc.*	*cc.*	*cc.*		
For antigen.								
1	0.2	0.2					+++	++++
For precipitin.								
2	0.2		1 : 10	0.2			++	++
3	0.2		1 : 100	0.2			+	+
4	0.2		1 : 500	0.2			+	+
5	0.2		1 : 1,000	0.2			+	+
6	0.2		1 : 5,000	0.2			+	+
7	0.2		1 : 10,000	0.2			o	±
Controls.								
8	0.2				0.2		o	Slight precipitate (spontaneous).
9			1 : 10	0.2		0.2	o	o
10			1 : 10	0.2	0.2		o	o
11	0.2					0.2	o	o
12		0.2				0.2	o	o
13		0.2	1 : 25,000	0.2			+	+

Tube 1 shows that the serum contained a considerable amount of antigen, while Tubes 6 and 7 show that the precipitin titer of the same serum equalled 1:10,000. Tubes 8 to 13 were used to show that no non-specific precipitation occurred in any of the components of the primary reaction. With the subsequent tests, this series of controls was repeated invariably. Their results were always as anticipated and they will be omitted in detail from the following protocols.

The batches of Serum 25, kept in the ice box, showed definite spontaneous precipitation at the end of 24 hours.

July 28. Titration of the antigen and antibody content of this serum was carried out as follows.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	cc.		cc.		
For antigen.							
1	Undiluted.	0.1	0.1			++	++++
2	1 : 10	0.1	0.1			+	+++
3	1 : 100	0.1	0.1			+	++
4	1 : 250	0.1	0.1			+	+
5	1 : 500	0.1	0.1			o	±
6	1 : 1,000	0.1	0.1			o	o
For precipitin.							
7	Undiluted.	0.1		1 : 1,000	0.1	+	+
8	"	0.1		1 : 5,000	0.1	±	±
9	"	0.1		1 : 10,000	0.1	o	o

This series of tests shows that the quantity of antigen present in Serum 25 (after injection) was sufficient to give a visible reaction when diluted 500 times. At the same time the precipitin titer of this serum containing free antigen was 1 : 1,000. In the previous 24 hours, however, while spontaneous precipitation had occurred in the serum, the precipitin titer decreased from 1 : 5,000 to 1 : 1,000.

Spontaneous precipitation continued, and at the end of 48 hours the following proportions of egg albumin and its precipitin were found to be present.

July 29. Serum 25 titrated as follows:

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*		*cc.*		
For antigen.							
1	Undiluted.	0.1	0.1			++	+++
2	1 : 10	0.1	0.1			+	++
3	1 : 100	0.1	0.1			±	+
4	1 : 250	0.1	0.1			o	+
5	1 : 500	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 500	0.1	+	+
7	"	0.1		1 : 1,000	0.1	o	o
8	"	0.1		1 : 5,000	0.1	o	o

July 31. 96 hours after the serum had been obtained, the tubes were turbid with considerable flocculated material in the sediment. The turbidity disappeared on warming the serum, but the sediment remained, giving evidence of the specific nature of this spontaneous precipitate. Titration of the serum at the end of 96 hours gave the following values for its content of free antigen and antibody.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*		*cc.*		
For antigen.							
1	Undiluted.	0.1	0.1			+++	+++
2	1 : 10	0.1	0.1			++	++
3	1 : 150	0.1	0.1			+	+
4	1 : 200	0.1	0.1			+	+
5	1 : 300	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 100	0.1	±	+
7	"	0.1		1 : 350	0.1	o	o

This shows that during the course of spontaneous precipitation in the serum, both egg albumin and its precipitin became decreased. At the end of 96 hours the antigen titer had fallen to 1 : 200 and the precipitin titer to 1 : 100.

Subsequent titrations were carried out to follow the decrease of these anti-substances coincident with progress of spontaneous precipitation in their serum.

At the end of 144 hours the serum no longer contained precipitin, while the antigen titer had decreased to 1 : 100.

At the end of 196 hours the precipitin was still zero, while the antigen titer remained constant at 1 : 100.

The results of these titrations of the supernatant serum during spontaneous precipitation are summarized in Table II, and represented graphically in the curve of the reaction (Text-fig. 1).

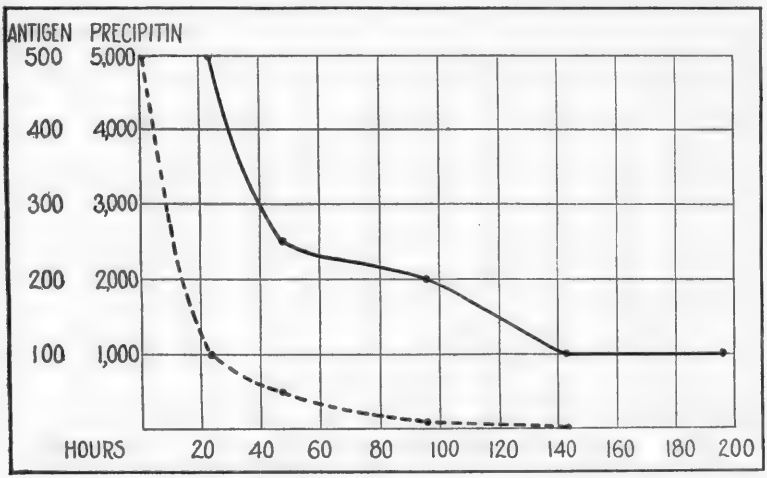
TABLE II.

Summary of Experiment VI.

Hours.	Precipitin.	Antigen.
2	1 : 5,000	?
24	1 : 1,000	1 : 500
48	1 : 500	1 : 250
96	1 : 100	1 : 200
144	0	1 : 100
196	0	1 : 100

The chart of the observed amounts of antigen and antibody remaining dissociated in the supernatant fluid of the precipitin reaction suggests at once that the process has taken place according to a definite law. As the amounts of antigen and precipitin are stated only in terms of the dilution of mixtures whose protein content was not accurately estimated it is not possible to analyze this curve in detail. Experiments are being undertaken, therefore, to determine quantitatively the mutual relationship of the factors of this reaction. While, however, calculations are not possible which would reveal the nature of this reaction, it is permissible to point out that the curve is not unlike that of some colloidal precipitations. In this case, the amount of precipitate together with the amount of precipitin remaining in solution in the presence of the precipitable substance is apparently a function of the concentration of the precipitable substance; *e.g.*, crystalline egg albumin, the colloidal properties of which are indisputable. Although the experiment was not conducted upon a quantitative basis, the regularity of the curve of its results is striking. From this feature it is a fair assumption to suppose that the reacting

antibodies were strictly homologous, and that the absence of irregularity in the curve is evidence that the antigen was a relatively simple substance. This confirms the chemical evidence of the purity of the preparation of crystalline egg albumin used as antigen.



—— Antigen (egg albumin).
- - - - - Precipitin.

TEXT-FIG. 1. Graphic representation of the results of Experiment VI, showing the changes in the quantities of antigen and antibody during spontaneous precipitation in a serum containing these substances. The quantities are expressed by titration values.

SUMMARY.

1. In these studies several phases of the precipitin reactions were investigated by the use of purified proteins as antigens. These preparations were edestin from hemp-seed and crystalline ovalbumin from fresh eggs. The ovalbumin, isolated by the method of Hopkins and Pinkus, was apparently as pure as is obtainable by chemical means. This albumin, however, produced moderately severe anaphylactic reactions in animals sensitized with ovoglobulin. Anaphylactic tests of the individuality of a protein cannot be any longer regarded as the criterion of the purity of the substance as an antigen.

Wells and Osborne[16] have shown that proteins of considerable chemical difference may have a common antigenic group which causes mutual anaphylactic reactions in animals sensitized to these proteins. In particular, as egg globulin is a mixture of proteins, one of which is undoubtedly egg albumin, anaphylaxis produced by injections of albumin into animals sensitized to the so called globulin offers no evidence for or against the purity of the albumin. The character of the curves shown in Text-fig. 1 confirms the assumption, based upon chemical data, that crystalline egg albumin is a single protein.

2. With edestin and crystalline egg albumin as antigens, phases in the precipitin reaction were found in which these substances and their specific precipitins could be demonstrated to be coexistent but ununited in the same serum.

3. When edestin or crystalline egg albumin is injected into a rabbit immunized thereto, the antigen may be found in the circulating blood during 48 hours after its injection, while at the same time the animal maintains a high titer of free precipitin in its blood.

4. When the pure protein antigen is mixed in proper proportions with the serum of a specifically immunized rabbit and the resulting precipitate removed by centrifugation, the supernatant fluid contains both antigen and antibody.

5. The serum drawn from a rabbit during the period in which free antigen and antibody are coexistent in the circulation undergoes slow spontaneous precipitation when kept in sterile tubes in the ice box. The curve of this reaction is reproduced as Text-fig. 1. The relationships of the parabola indicate that the interaction of antigen and antibody takes place according to a definite law. When sufficient quantitative data are obtained to allow an analysis of this curve, the formulas for this reaction will undoubtedly throw light upon the chemical or physical nature of the process.

6. The protective action of the solution of egg albumin as a third colloid preventing precipitation in a reaction between human serum and its antibody was readily demonstrated. This observation and the constancy of the long prozone in precipitin test with egg albumin are in accord with the protective action of ovalbumin upon colloidal gold.

THE FOCAL PULMONARY TUBERCULOSIS OF CHILDREN AND ADULTS.

By EUGENE L. OPIE, M.D.

(*From the Pathological Laboratory of Washington University Medical School, St. Louis.*)

PLATES 68 TO 71.

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In order to obtain data concerning the relation of phthisis to tuberculous infection during childhood it has been desirable to determine the local frequency of infection among both children and adults. In a city such as St. Louis conditions of living differ widely from those in cities of the old world where the incidence of tuberculosis has heretofore been studied.

Statistics which have been obtained would be of little value if they had not demonstrated that here as in European cities tuberculous infection is well nigh universal among those who have reached adult life. A detailed examination of the localization and character of the lesions found in the lungs and elsewhere has furnished new data concerning tuberculous infection, for it has demonstrated that the healed or healing tuberculous lesions demonstrable in the lungs of many children and of nearly all adults have characters which serve to distinguish them from the progressive pulmonary tuberculosis of adults. They are identical with the tuberculosis of infancy and early childhood and exhibit little resemblance to the phthisis of adults.

The use of x-ray plates made from the lungs has proven an efficient means of determining the presence of healed tuberculous lesions within the substance of the organs. Since calcium salts are impervious to the x-rays very small calcified nodules may be readily identified in the plate (Figs. 1 to 9). Partially calcified foci containing caseous material of soft friable consistence are conspicuous in x-ray plates. Search for tuberculous lesions has been made in the lung hardened in formalin and, as far as necessary, cut into thin sections. In no in-

stance has the x-ray shadow been used as evidence of the presence of a calcified nodule. The difficulty of finding a nodule well depicted in the plate has often been great (Fig. 8), and in a number of instances no lesions would have been demonstrable without the aid of the plate. In one specimen a small intrapulmonary nodule obvious in the plate was long sought by careful palpation of sections from 0.5 to 1 cm. in thickness, and finally found within the angle at the bifurcation of a bronchus of medium size.

Lesions characterized by the presence of nodules which had undergone caseation were regarded as tuberculous. A calcified nodule surrounded by a fibrous capsule was regarded as a tuberculous lesion in which calcium had been deposited in caseous material. With few exceptions calcified nodules were found both within the substance of the lung and in regional lymphatic nodes at the hilus of that lobe in which the pulmonary lesion occurred. Although it is well known that tuberculous lesions in healing may form fibrous scars, fibrous nodules were not classified as tuberculous even when they formed wedge-shaped masses penetrating from the pleura into the substance of the apices. Wherever there was doubt concerning the nature of a lesion microscopic examination was made. In two instances tubercles were found by microscopic examination although none were recognized by the naked eye.

Pulmonary tuberculosis in infancy and early childhood is accom panied by enlargement and caseation of intrapulmonary bronchial and of tracheal lymph nodes. The pulmonary lesion may be situated in any part of the lungs and is not more frequently localized in the apices than elsewhere; there is indeed a tendency for the lesion to occupy the mid-part of either lung, frequently affecting the middle lobe on the right side or the corresponding part of the upper lobe on the left. There is in infancy little formation of fibrous tissue about the tuberculous lesion, but with increasing age encapsulation by fibrous tissue occurs more frequently. Cavities are much less frequently formed in children than in adults and usually occupy only a small part of the tuberculous lesion. Dissemination of tuberculosis frequently occurs and tuberculosis of the meninges, spleen, or other tissue is frequently associated with pulmonary tuberculosis and tuberculosis of bronchial lymphatic nodes.

Pulmonary tuberculosis of adults almost invariably affects the apices of the lungs. The disease tends to pursue a chronic course; there is often abundant formation of fibrous tissue and cavities are commonly present when the lesion is advanced. The disease does not implicate the regional lymphatic nodes at the hilus of the diseased lung. In the absence of acute miliary tuberculosis generalization with tuberculosis of the meninges, spleen, or other organs is uncommon unless the disease is approaching a fatal termination.

For the purpose of the present investigation the pulmonary tuberculous lesions which occur in childhood have been compared with those of adults. The age of 18 years has been arbitrarily selected as the line of division between adolescence and adult life. During the period within which autopsies were made upon fifty adults ninety-three children were examined. Tuberculosis was the cause of death in eleven children and in three adults; in the bodies of those who had died of other diseases, tuberculosis was found eleven times in children and forty-seven times in adults.

The incidence of tuberculosis at different ages is shown in Table I.

TABLE I.

Age.	No. of autopsies.	Tuberculosis.			
		Present.	Fatal.	Non-fatal.	In those who have died with other diseases.
Children.					
yrs.			*per cent*			*per cent*
Under 1	43	4	9.3	4	0	0
1– 2	16	1	6.2	1	0	0
2– 5	14	6	42.8	3	3	27.3
5–10	11	5	45.5	2	3	33.3
10–18	9	6	66.7	1	5	62.5
Adults.					
18–30	6	6	100	1	5	100
30–50	23	23	100	1	22	100
50–70	15	15	100	1	14	100
70+	6	6	100	0	6	100

The proportion of instances of fatal tuberculosis in Table I is of little significance since it depends upon the frequency with which tuberculosis is admitted to the two hospitals from which autopsies were obtained. Since all diseases are admitted to the St. Louis Children's Hospital the figures for children represent approximately the frequency of the disease in this city. Recognized tuberculosis is not usually admitted to the Barnes Hospital. When fatal tuberculosis is excluded the figures representing the incidence of tuberculous infection among those who have died of other diseases are an index of the frequency of infection in the general population. In most instances the anatomical characters of the lesion indicate that it has long existed in the body and bears no relation to the fatal illness. Before the age of 2 years the disease is almost always fatal, but later there is a progressive increase in the number of infected individuals. Evidence of infection has been found in all adults.

The result of this incidental study of the occurrence of tuberculosis in a small number of autopsies is in accord with the studies of Naegeli made in Zurich and of Burkhardt made in Dresden upon a far larger number of autopsies. Among adults Naegeli[1] found tuberculosis in 97 per cent and Burkhardt[2] in 90 per cent of all individuals.

A survey of tuberculosis which has been found furnishes a satisfactory basis for comparison between the lesions of infants and adolescents on the one hand and of adults on the other. The well known characters which distinguish the tuberculosis of childhood will be cited briefly.

Tuberculosis of Children.

Location and Character of the Pulmonary Lesions of Childhood.—Tuberculosis of infancy has occurred five times and in all instances was fatal within the 1st or 2nd year of life (Text-fig. 1 and Fig. 1). Massive caseous lesions in some instances have implicated a considerable part of one lung but do not affect the apex more frequently than other parts. The middle lobe on the right side in one instance has been almost wholly caseous. Small cavities are occasionally formed. These lesions have exhibited no tendency to heal. After

[1] Naegeli, O., *Virchows Arch. path. Anat.*, 1900, clx, 426.
[2] Burkhardt, A., *Z. Hyg. u. Infectionskrankh.*, 1906, liii, 139.

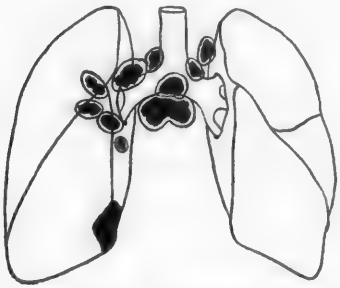
the end of the 2nd year tuberculous lesions have usually been small, seldom more than 1.5 cm. in diameter, and may be situated in any part of the lung substance (Text-figs. 2 to 6). They may be designated focal lesions and differ from the lesions of infancy only in size. A tendency to heal may be indicated by encapsulation of the caseous focus with fibrous tissue. In one instance an encapsulated caseous nodule has been found in a child only 2 years of age. The caseous material undergoes calcification and at first assumes a dry friable mortar-like consistency; later it is converted into material of stony hardness. Nodules consisting of a calcified center surrounded by a fibrous capsule and less than 1 cm. in diameter have been found in a considerable proportion of all children between the ages of 4 and 15 years (Figs. 2 and 3). More than two-thirds of all these tuberculous foci have been in contact with the pleural surface of the lung (Text-figs. 1, 4, and 6).

The study of Ghon[3] based upon a large number of observations has demonstrated the distribution of pulmonary tuberculous infection in children. The distribution of the lesion in the cases which I have studied is cited in order that the lesions of children and of adults may be compared. In most instances there has been only one focus of pulmonary tuberculosis (Text-figs. 1, 2, and 5); smaller nodules nearby may be obviously secondary to a larger lesion (Text-fig. 3). In three instances there have been two or more separate foci of infection (Text-fig. 6). The distribution of these lesions in the various lobes of the lung have been as follows: right upper lobe, 7; right middle lobe, 3; right lower lobe, 5; left upper lobe, 5; left lower lobe, 5. These figures are cited to show that the tuberculous lesions of childhood are impartially scattered in the substance of the lung. In one instance in a child 11 years of age tuberculosis had the usual characters of the phthisis of adults; the apices of both lungs were affected and cavities of considerable size occurred in both lungs. It is well known that fatal tuberculosis of this type is not uncommon after the 6th or 7th year.

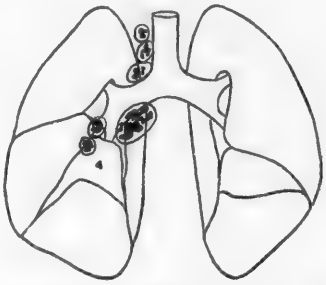
Relation of the Pulmonary Focus to Lesions in Regional Lymphatic Nodes.—The most conspicuous character of tuberculosis in children

[3] Ghon, A., Der primäre Lungenherd bei der Tuberkulose der Kinder, Berlin, 1912.

is the extent of involvement of regional lymphatic nodes. In nineteen instances caseous or calcified tuberculous lesions were found both in the substance of the lung and in the lymphatic nodes either

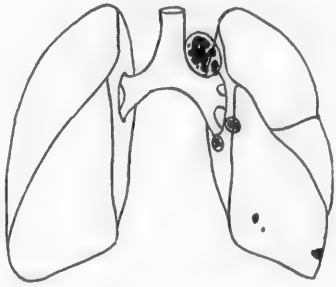


TEXT-FIG. 1. Posterior view of the lungs of a child, age 9 months, who died of pulmonary tuberculosis, meningitis, and general miliary tuberculosis. There is a caseous focus in the left lower lobe and tuberculosis of the regional lymphatic nodes. Compare with Fig. 1 showing the same lungs.



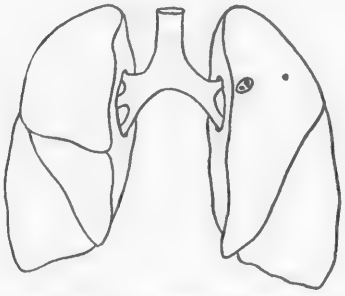
TEXT-FIG. 2. Median view of the lungs of a child, age 2½ years, who died of ileocolitis. There is a caseous focus in the right lower lobe and caseation of the regional lymphatic nodes.

TEXT-FIG. 3. Anterior view of the lungs of a child, age 3 years, who died of postdiphtheritic paralysis and bronchopneumonia. There is a caseous focus surrounded by tubercles in the left upper lobe and caseation of the regional lymphatic nodes.

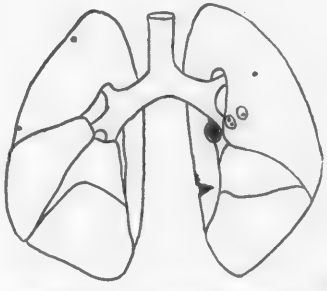


TEXT-FIG. 4. Posterior view of the lungs of a child, age 6 years, who died of otitis media and acute suppurative meningitis. There are three calcified nodules in the lower right lobe and in the regional lymphatic nodes. Compare with Fig. 2 from the same lungs.

within the lung near its hilus or in the adjacent peribronchial or tracheal nodes. In only one instance has a lesion been found in the lymphatic nodes although none was demonstrable in the substance of the lung. It is not improbable that small lesions may be overlooked. If caseation has failed to occur complete resolution of the lesion is possible. In one instance a tuberculous focus was found in the lung although none was found in the adjacent lymphatic nodes. In the child with apical tuberculosis mentioned above, the regional lymphatic nodes were enlarged and contained tubercles but were not caseous.



TEXT-FIG. 5. Anterior view of the lungs of a child, age 10 years, who died of osteomyelitis and pyemia. There is an encapsulated, caseous, and partly calcified nodule in the left upper lobe and a lymphatic node nearby contains encapsulated caseous nodules.



TEXT-FIG. 6. Median view of the lungs of a child, age 15 years, who died of an abscess of the brain and bronchopneumonia. There are calcified foci in the left upper and lower lobes and calcification of regional lymphatic nodes. In the right upper lobe there are encapsulated caseous foci but no caseation of the lymph nodes on this side. Compare with Fig. 3 from the same lungs.

Ghon, studying the tuberculosis of children, has shown the almost constant association of pulmonary lesions and lesions of the pulmonary and bronchial nodes. He has found that the pulmonary lesion often exhibits characters which indicate that it is older than the lesion of the adjacent lymphatic nodes and this observation he believes furnishes evidence that the pulmonary lesion is primary and the lymphatic foci are secondary. In several instances the specimens which

I have examined have exhibited a similar relation between pulmonary and lymphatic lesions; the lesion of the lung has been encapsulated whereas there has been no encapsulation of the lymphatic lesion.

The susceptibility of the lymphatic nodes of infants and very young children to tuberculosis is well shown by great enlargement and widespread caseation of the affected nodes. Masses of greatly enlarged almost wholly caseous lymphatic nodes occupy the hilus of the lung in which the pulmonary focus is situated, and similar masses fill the spaces between trachea and lung or the angle at the bifurcation of the trachea (Text-figs. 1 to 4). At a more advanced age there is less enlargement and caseation of regional lymphatic nodes and in children between the ages of 10 and 18 years it is not uncommon to find small caseous or calcified nodules in only one or two lymphatic nodes (Text-figs. 5 and 6).

Tuberculosis of Adults.

Table I shows that tuberculosis has been found in all of fifty individuals above the age of 18 years. The criteria previously described have been kept in mind and no lesions regarded as doubtful have been recorded as tuberculous. Tuberculous lesions characterized by the presence of fresh tubercles have been designated active tuberculosis. Lesions in which caseous foci are completely surrounded by fibrous tissue have been found much more frequently; in this group are included a considerable number of lesions in which the caseous material has a dry mortar-like consistence, is obviously partially calcified, and gives a conspicuous shadow upon the x-ray plate. Lesions in which a firmly calcified center is surrounded by a capsule of fibrous tissue have been classified as healed tuberculosis. Table II shows the frequency of active, encapsulated, and healed tuberculosis in the series of autopsies which have been studied. When active and encapsulated lesions occurred in the same individual the process has been listed as active; when encapsulated and calcified lesions occurred together the disease was classified as encapsulated. Tuberculosis was regarded as healed only when all the lesions were firmly calcified and surrounded by fibrous tissue.

TABLE II.

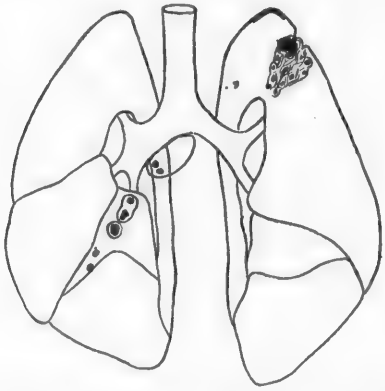
Age.	No. of autopsies.	Active tuberculosis.		Encapsulated caseous lesions.		Healed tuberculosis.	
				Children.			
yrs.			*per cent*		*per cent*		*per cent*
Under 1	43	4	9.3	0	0	0	0
1– 2	16	1	6.2	0	0	0	0
2– 5	14	5	35.7	1	7.1	0	0
5–10	11	2	18.2	1	9.1	2	18.2
10–18	9	1	11.1	4	44.4	1	11.1
				Adults.			
18–30	6	1	16.7	3	50.0	2	33.3
30–50	23	2	8.7	12	52.2	9	39.1
50–70	15	2	13.3	7	46.7	6	40.0
70+	6	1	16.6	3	50.0	2	33.3

The table shows that active tuberculosis is very frequent between the ages of 2 and 10 years. These lesions are often of small size and undergo encapsulation. Latent or encapsulated tuberculosis is found in approximately half of all individuals over 10 years of age. Many of these lesions heal completely and a third of all adults have firmly calcified foci which represent former tuberculous infection.

The frequency of healed tuberculosis in the autopsies which I have studied is less than that found by Naegeli and by Burkhardt, and the proportion of latent encapsulated tuberculosis is greater. I have not recorded the pulmonary lesion as healed unless it has been compactly calcified and have excluded from the group of healed lesions cases in which caseous tuberculosis was found in regional lymphatic nodes.

Tuberculous lesions of the lungs may be separated into two sharply definable groups: (1) Lesions occupying the apices of the lungs, tending to spread diffusely through the tissue of the apex and unaccompanied by caseation (or calcification) of the regional lymphatic nodes represent what is generally regarded as the usual type of tuberculosis of adults (Text-figs. 7 and 8). This lesion which is often fatal may undergo complete healing and in a considerable proportion of autopsies upon individuals who have presented no evidence of

TEXT-FIG. 7. Posterior view of the lungs of a negro, age 39 years, who died of aneurysm of the aorta. There are two calcified nodules in the lower left lobe and similar nodules in the regional lymphatic nodes. There is a diffuse tuberculous lesion with caseation and fibrosis at the apex of the right lung. There is no caseation of the lymphatic nodes on the right side.



TEXT-FIG. 8. Median view of the lungs of a white man, age 74 years, who died of general arteriosclerosis, chronic diffuse nephritis, and fibrous myocarditis. There are calcified nodules in the right lower lobe and calcified and caseous foci in the regional lymphatic nodes. Near the apex of the left upper lobe is a diffuse tuberculous lesion with caseation and fibrosis. There is no caseation of the lymphatic nodes on the left side.

pulmonary tuberculosis the apex of one or both lungs is occupied by an area of diffuse fibrous induration within which occur caseous or calcified nodules. For convenience I shall designate this lesion apical tuberculosis. (2) The second group consists of circumscribed lesions situated in any part of the lung, not more frequently in the upper parts of the upper lobes than elsewhere, and accompanied by similar caseous or calcified lesions of the regional lymphatic nodes (Text-figs. 9 to 20). In the lungs of adults who have died with disease other than tuberculosis this is the commonest type of tuberculosis. These lesions have the distribution and characters of the circumscribed tuberculous lesions which begin to appear after the 2nd year of life and during later childhood are often found to be encapsulated and even calcified. They may be designated focal tuberculosis.

The relative frequency of the two types of pulmonary tuberculosis in the present series of autopsies is shown in Table III.

TABLE III.

Age.	No. of autopsies.	Focal pulmonary tuberculosis.		Apical tuberculosis.	
		Children.			
yrs.			*per cent*		*per cent*
Under 1	43	4	9.3	0	0
1– 2	16	1	6.2	0	0
2– 5	14	6	42.8	0	0
5–10	11	5	45.5	0	0
10–18	9	5	55.5	1	11.1
		Adults.			
18–30	6	5	83.3	1	16.7
30–50	23	21	91.3	3	13.0
50–70	15	14	93.3	4	26.7
70+	6	6	100.0	3	50.0

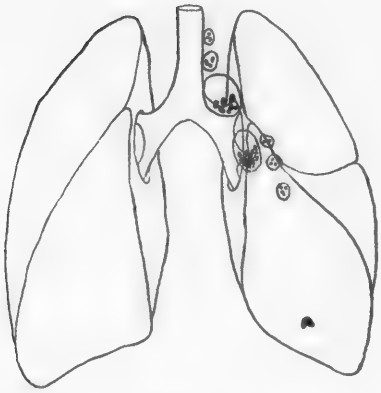
It is noteworthy that the incidence of focal tuberculosis increases continuously after the 2nd year of life. Comparison with Table I shows that the focal type of tuberculosis is frequently fatal in early childhood whereas after the 10th year it is rarely a cause of death. At the age of 18 a large proportion of all individuals have acquired these focal lesions but a few have escaped; at least 92 per cent of all

adults possess focal lesions. Apical phthisis in the present series of autopsies makes its appearance at the age of 11 years and subsequently apical lesions increase in frequency from adolescence to old age. The existence of focal lesions on the one hand does not preclude the occurrence of apical tuberculosis. On the other hand, in one instance of apical tuberculosis between the 18th and 30th years there was no focal lesion and apical tuberculosis was associated with caseous tuberculosis of the regional lymphatic nodes and general miliary tuberculosis. I shall discuss the relation of focal to apical tuberculosis in a subsequent paper.

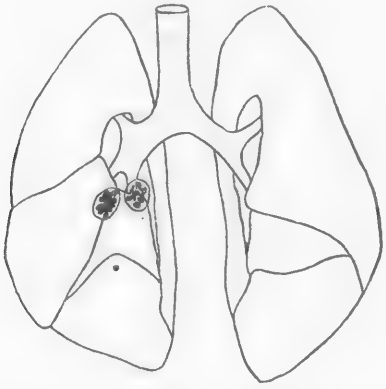
Location and Characters of the Focal Tuberculosis of Adults.—The circumscribed tuberculous foci which occur in the lungs of adults do not differ in character and distribution from those which are found in older children. They rarely exceed 1 cm. in diameter and the smaller nodules perhaps not more than 1 mm. across are found with much difficulty even when they are calcified and cast a conspicuous shadow upon the x-ray plate. Attention is often first attracted to these pulmonary nodules by the more conspicuous lesions almost always present in the regional lymphatic nodes.

In the greater number of lungs there is only one focus of tuberculous infection (Text-figs. 9 to 12); when small nodules are near a relatively large focus they have been regarded as secondary to it (Text-figs. 13 and 14). In about a third of the autopsies studied two foci of pulmonary infection have been present (Text-figs. 15 to 18), and in several instances three (Text-fig. 19), four, or more foci have been found. In one instance (Text-fig. 20 and Fig. 9) in the absence of any evidence of healed miliary tuberculosis in other organs a great number of firmly calcified nodules varying in size were sprinkled throughout the lungs so that every lobe contained numerous foci of infection.

The distribution of focal tuberculous lesions in the lobes of the lung has been as follows: right upper lobe, 17; right middle lobe 5; right lower lobe 15; left upper lobe 14; left lower lobe 15. The distribution of these lesions in the adult lung is almost identical with that in the lungs of children and occurs in the different lobes in approximate proportion to their volume. Nearly one-half of these focal lesions are situated immediately below the pleural surface (Text-

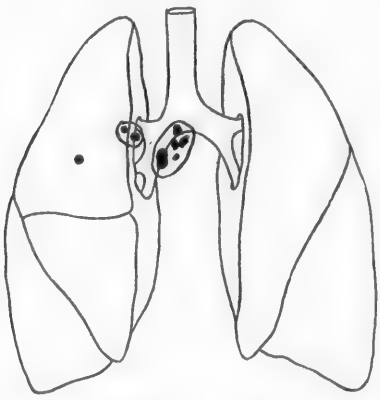


TEXT-FIG. 9. Posterior view of the lungs of a white man, age 19 years, who died of acromegaly, adenoma of the hypophysis, and bronchopneumonia. There is an encapsulated caseous focus in the right lower lobe and similar nodules in the regional lymphatic nodes.

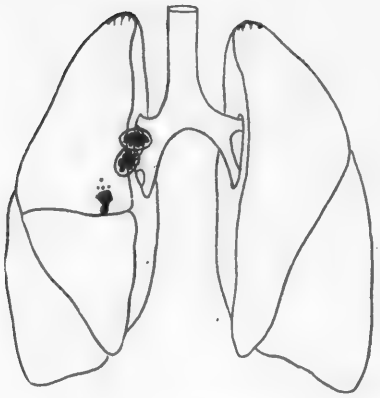


TEXT-FIG. 10. Median view of the lungs of a white man, age 35 years, who died of Hodgkin's disease, chronic diffuse nephritis, and cirrhosis of the liver. There are encapsulated, partially calcified caseous foci in the lower right lobe and in adjacent lymphatic nodes.

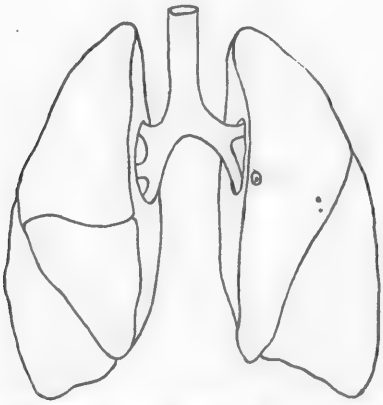
TEXT-FIG. 11. Median view of the lungs of a white woman, age 18 years, who died of typhoid fever. There are encapsulated, partially calcified, caseous foci in the right upper lobe and in adjacent lymphatic nodes.



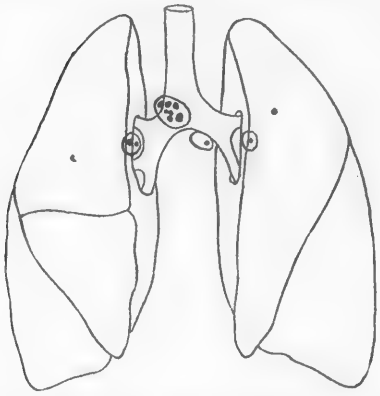
TEXT-FIG. 12. Anterior view of the lungs of a white woman, age 30 years, who died of Hodgkin's disease. There are encapsulated nodules in the right upper lobe and in adjacent lymphatic nodes.



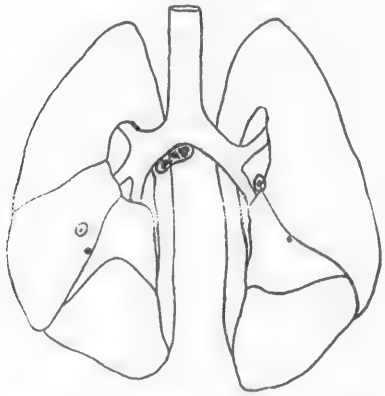
TEXT-FIG. 13. Anterior view of the lungs of a white woman, age 34 years, who died of Addison's disease, and tuberculosis of the adrenal glands, spleen, and left Fallopian tube. There are encapsulated, partially calcified, caseous nodules in the right upper lobe and in adjacent lymphatic nodes. These lesions appear to be older than those of the adrenal and spleen, etc. Compare with Fig. 4.



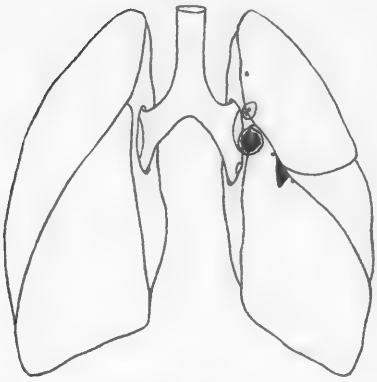
TEXT-FIG. 14. Anterior view of the lungs of a white man, age 56 years, who died of general arteriosclerosis, small granular kidneys, and hypertrophy of the heart. Inconspicuous calcified nodules occur in the left upper lobe and in a lymphatic node. Compare with Fig. 7.



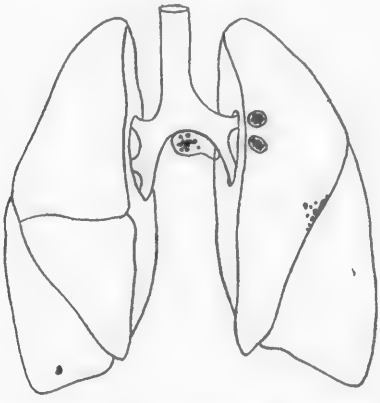
TEXT-FIG. 15. Anterior view of the lungs of a white man, age 71 years, who died of general arteriosclerosis, small granular kidneys, and hypertrophy and dilatation of the heart. There are two calcified nodules in the lungs and calcified and encapsulated caseous lesions in the lymphatic nodes.



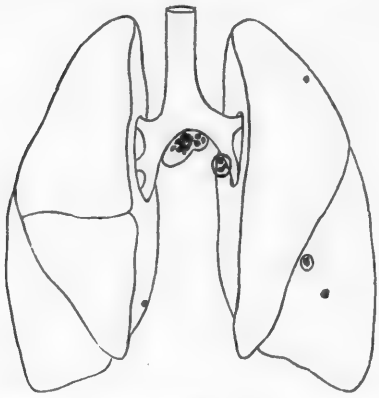
TEXT-FIG. 16. Median view of the lungs of a white woman, age 48 years, who died following an operation for carcinoma of the cervix uteri. There are calcified and encapsulated caseous nodules in the lungs and in the lymphatic nodes. Compare with Fig. 5.



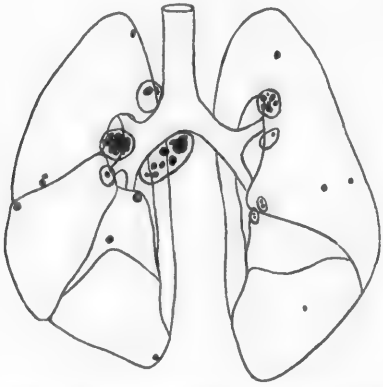
TEXT-FIG. 17. Posterior view of the lungs of a white man, age 80 years, who died of cirrhosis of the liver and chronic endocarditis. There is a calcified mass below the pleura of the right lower lobe and small calcified nodules occur in the pleura nearby. There is a calcified nodule in the right upper lobe and the lymphatic nodes at the hilus of the right lung are partially calcified.



TEXT-FIG. 18. Anterior view of the lungs of a white man, age 46 years, who died of æstivo-autumnal malaria. There is a group of calcified nodules in contact with the interlobular pleura of the left upper lobe. There is a calcified nodule in the right lower lobe. There are partially calcified lymphatic nodes at the hilus of the left lung but none on the right side. Compare with Fig. 6 from the same lungs.



TEXT-FIG. 19. Anterior view of the lungs of a white woman, age 50 years, who died of small granular kidneys, arteriosclerosis, and dysentery. There are multiple calcified nodules in the lungs and similar lesions in the lymphatic nodes. Compare with Fig. 8 from the same lungs.



TEXT-FIG. 20. Median view of the lungs of a negress, age 36 years, who died following panhysterectomy for myomata of the uterus. There are multiple calcified nodules in all the lobes and numerous partially calcified lymphatic nodes. Compare with Fig. 9 from the same lungs. The text-figure shows only those nodules which were identified in the lung. The x-ray plate shows the presence of a far larger number of calcified nodules.

figs. 13, 17, 18, 20, etc.). It is not uncommon to find a calcified nodule immediately below the puckered pleura and about it upon the adjacent pleura a group of small nodules (Text-figs. 17 and 18 and Fig. 6). Fibrous adhesions usually bind together the adjacent pleural surfaces.

In children freshly caseous focal lesions are predominant, whereas in adults encapsulated or calcified nodules are much more common. The following classification serves to indicate the character of the focal lesions which were found in adults: freshly caseous, 1; caseous and encapsulated, 5; caseous and encapsulated with beginning deposit of calcium salts producing dry chalky or mortar-like material, 12; firmly calcified, 48. In two instances tuberculous foci were found in the substance of the lung but none were found in lymphatic nodes.

Relation of the Pulmonary Tuberculous Focus to Tuberculosis of the Regional Lymphatic Nodes.—When a localized focus of active or preexisting tuberculous infection has been found in one of the lobes of the lung a similar lesion, with few exceptions, has been found in intrapulmonary lymphatic nodes nearby or in intra- or extrapulmonary nodes near the hilus of the lobe affected (Text-figs. 9 to 20). In instances in which pulmonary lesions occurred in more than one lobe tuberculous lesions have been usually found in regional lymphatic nodes corresponding to each of the affected lobes (Text-figs. 15 to 17); but occasionally with multiple lesions some were unaccompanied by lesions of the adjacent lymphatic nodes (Text-figs. 18 and 19). The lesion of the lymphatic node is usually larger and more readily found than the pulmonary lesion and frequently there is a chain of tuberculous nodes beginning in the lung and extending along the corresponding bronchus to one or the other side of the trachea (Text-figs. 9 and 11). In correspondence with the distribution of tuberculosis often seen in childhood a large mass of nodes containing calcified nodules is found in adults at the bifurcation of the trachea and in the spaces above the bronchi between the trachea and lung (Text-figs. 9, 11, 12, 18, 19, and 20). Text-figs. 7 to 20 and Figs. 4 to 9 indicate the size and distribution of the lesions of the lymphatic nodes and their position in relation to the pulmonary lesion. In most instances irregular calcified masses occur within indurated lymphatic nodes deeply pigmented with carbon particles. In other instances

spherical nodules 2 or 3 mm. in diameter consisting of a caseous or partially calcified mortar-like center and a thick fibrous capsule are scattered within the substance of enlarged pigmented lymphatic nodes. Freshly caseous lesions are rarely seen in adults. The lesion of the regional lymphatic node is usually similar to that within the lung, both being either caseous or calcified, but in eight instances the pulmonary focus was firmly calcified whereas the lymphatic lesion was caseous. In two instances this relation was reversed.

Of the tuberculous lesions which have occurred in the lymphatic nodes and are unaccompanied by lesions of the lung two consist of typical gray fibroid tubercles identified by microscopic examination and three are calcified nodules. In one specimen a large lymphatic node at the bifurcation of the trachea contains firmly calcified foci: in a second a minute calcified nodule is found with the aid of an x-ray plate in a lymphatic node near the hilus of the left upper lobe and a minute shadow of similar size occurs in the lung substance nearby, but careful examination has failed to reveal a pulmonary lesion. In a third instance a calcified nodule surrounded by a fibrous capsule 4 mm. in diameter occurs in a lymphatic node below the bifurcation of the trachea and a second similar nodule occurs in a lymph node above the left bronchus; a pearly white nodule 2 mm. in diameter is found in the substance of the left lower lobe but there is no evidence of caseation or of calcification. The characters of these lesions suggest no doubt that they are identical with similar lesions found in association with tuberculous foci in the lung substance.

The foregoing observations have shown that tuberculous infection is practically universal. Dissemination of the disease among adults is so widespread that readily recognizable tuberculous lesions have been found in all of fifty individuals above the age of 18 years. First infection in almost all of those who reach adult life occurs in childhood and has the characters of a first infection in animals since it tends to implicate regional lymphatic nodes. Koch showed that a second infection of an animal already tuberculous shows greater tendency to heal and does not extend to regional lymphatic nodes, and this observation has been confirmed by numerous observers. Apical tuberculosis usually exhibits the characters of a second infection, since it pursues a chronic course and is unaccompanied by tuberculosis of regional lymphatic nodes.

CONCLUSIONS.

Evidence of tuberculous infection has been found in the lungs of all of fifty adults who have been examined. Approximately one-half of all adults have encapsulated lesions of the lungs or bronchial lymphatic nodes, whereas in one-third pulmonary and lymphatic lesions are firmly calcified and completely healed.

Tuberculous pulmonary lesions of adults who have died of diseases other than tuberculosis are of two types: (1) apical tuberculosis similar to the usual type of fatal phthisis and unaccompanied by caseation of the regional lymphatic nodes; (2) focal tuberculosis not more commonly situated in the apices of the lungs than elsewhere and accompanied by caseation (or calcification) of the adjacent lymphatic nodes.

Focal pulmonary tuberculosis of adults is identical with the tuberculosis of childhood. It occurs in at least 92 per cent of all adults. It may be acquired between the ages of 2 and 10 years but in more than half of a lyn iduals (in this city) makes its appearance between the ages of 10 and 18 years.

Tuberculosis of children does not select the apices of the lungs, is accompanied by massive tuberculosis of regional lymphatic nodes, and exhibits the characters of tuberculosis in a freshly infected animal, whereas tuberculosis which occurs in the pulmonary apices of adults has the characters of a second infection. Almost all human beings are spontaneously "vaccinated" with tuberculosis before they reach adult life.

EXPLANATION OF PLATES.

PLATE 68.

FIG. 1. X-ray plate of the lungs of a child, age 9 months. The shadows correspond to caseous foci at the base of the lower left lobe and in the regional lymphatic nodes as shown in Text-fig. 1.

FIG. 2. X-ray plate of the lungs of a child, age 6 years, showing the calcified foci indicated in Text-fig. 4.

FIG. 3. X-ray plate of the left lung of a child, age 15 years, showing the calcified foci depicted in Text-fig 6.

PLATE 69.

FIG. 4. X-ray plate of the lungs of a white woman, age 34 years, who died of Addison's disease. The positions of the right and left lungs are reversed. Compare with Text-fig. 13 which shows the position of the tuberculous lesions indicated by shadows in the plate.

FIG. 5. X-ray plate of the lungs of a white woman, age 48 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 16 from the same lungs.

PLATE 70.

FIG. 6. X-ray plate of the lungs of a white man, age 46 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 18 from the same lungs.

FIG. 7. X-ray plate of the lungs of a white man, age 56 years, showing small calcified nodules which were found in the lungs with much difficulty. Compare with Text-fig. 14 from the same lungs.

PLATE 71.

FIG. 8. X-ray plate of the lungs of a white woman, age 50 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 19 from the same lungs.

FIG. 9. X-ray plate of the lungs of a negress, age 36 years, showing a large number of calcified nodules in both lungs and in lymphatic nodes. The positions of the right and left lungs are reversed. Compare with Text-fig. 20 from the same lungs.

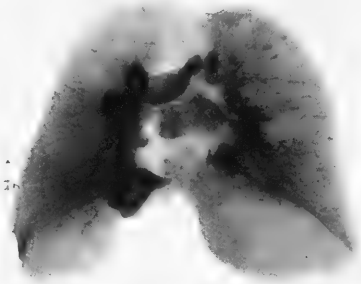


FIG. 1.

FIG. 2.

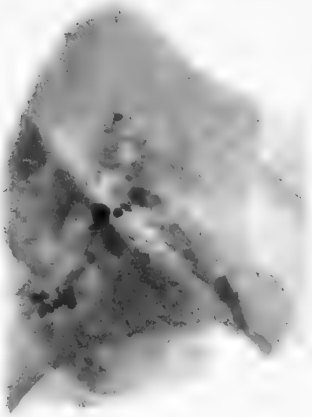


FIG. 3.

(Opie: Focal Pulmonary Tuberculosis.)

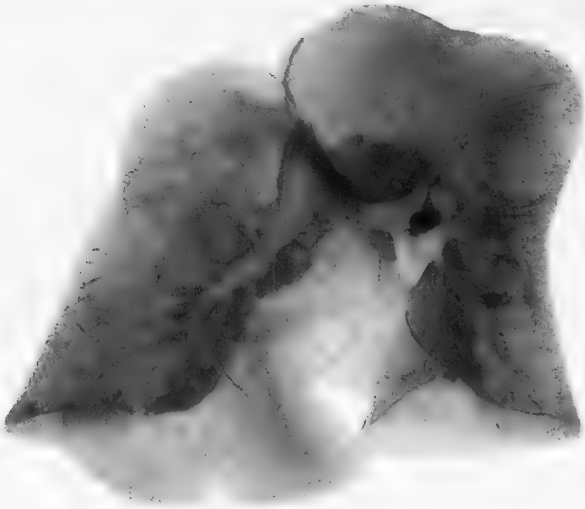


FIG. 4.

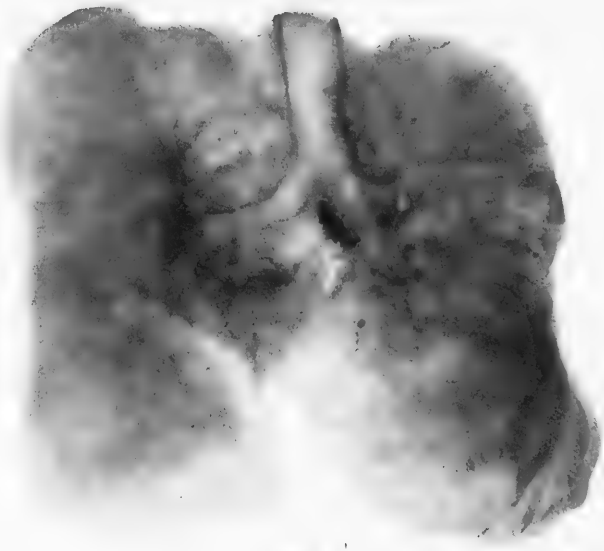


FIG. 5.

(Opie: Focal Pulmonary Tuberculosis.)

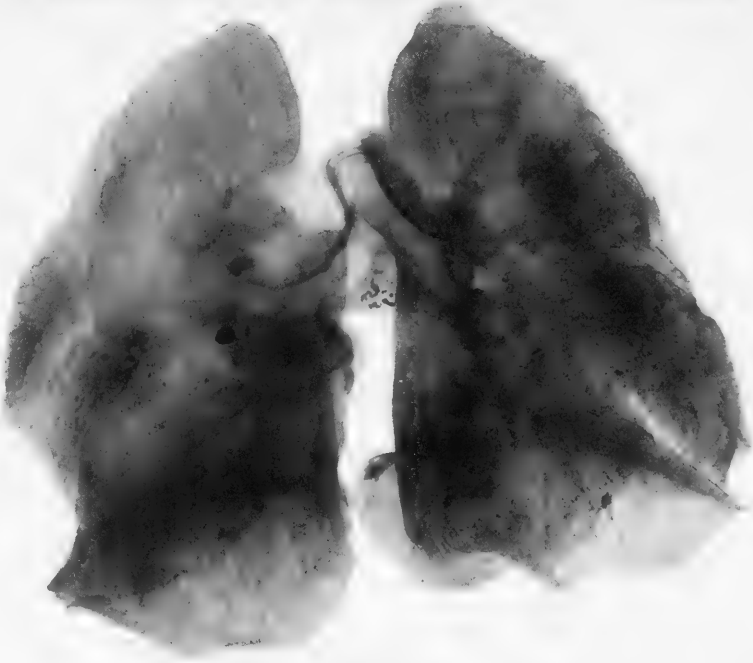


FIG. 6.

FIG. 7.

(Opie: Focal Pulmonary Tuberculosis.)

FIG. 8.

FIG. 9.

(Opie: Focal Pulmonary Tuberculosis.)

A CLASSIFICATION OF NON-HEMOLYTIC STREPTOCOCCI.

By RALPH A. KINSELLA, M.D., and HOMER F. SWIFT, M.D.

(*From the Medical Clinic of the Presbyterian Hospital, Columbia University, New York.*)

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No group of bacteria has been suspected of etiologic relationship with so many and diversified disease processes as the streptococcus. At the same time there is no group which suffers more confusion in the relationship of its numerous members. The purpose of the present work was to attempt a classification of twenty-eight strains of non-hemolytic streptococci by a comparative study of their biochemical and immunological reactions.

Since the streptococcus was first recognized as an infectious agent by Fehleisen (1) in 1882, various methods of classifying these organisms have been proposed. The object of every attempt to classify has been to determine whether all streptococci are identical with accidental differences in pathogenicity and cultural features or whether the differences noted constitute more or less fixed characteristics, which demand separate grouping for such members. All the schemes can be included in those dealing with morphology, the ability to produce special lesions, and immunological and biochemical reactions.

Classification on the basis of morphology was abandoned early because of the influence which cultural environment was found to have on morphology. The terms *longus* (2), *brevis*, and *conglomeratus* (3) are now considered merely descriptive of growth phenomena and not of fundamental properties.

The opinion that certain streptococci have a definite etiologic relationship to certain diseases was natural, since they were frequently associated with these diseases. Thus Fehleisen (1) assumed a certain variety to be the cause of erysipelas, while Rosenbach (4) was the first to call attention to the importance of the variety which he called *pyogenes*, in the production of local purulent processes. Similarly, scarlet fever and smallpox were regarded as being of streptococcus origin. This supposition, however, was weakened by the failure to establish

experimental proof of their etiologic rôle in these diseases, and further, by the finding of streptococci in the throat, gastro-intestinal tract, and feces of normal individuals. It might be noted that while formerly a special streptococcus was thought to be etiologically responsible in a given disease, many modern believers in the unity of streptococci prefer to regard them all as identical and their capacity of producing any given disease as merely an accidental and temporary property.

The relation of streptococcus to disease as well as the relationship of different streptococci to one another was at first based upon the results of agglutination reactions. Van de Welde (5), the first to study this reaction, reported (6) a specific agglutination between a univalent serum and its homologous streptococcus. Baginsky and Sommerfeld (7) found in the serum of scarlet fever patients specific agglutinins for streptococci isolated from the throats of such patients. Similar findings were reported by Hasenknopf and Salge (8) and Moser and von Pirquet (9). De Waele and Sugg (10) isolated streptococci from the blood of cases of smallpox both before and after death and found that the sera of smallpox patients agglutinated the variety of streptococcus obtained, while sera from unvaccinated individuals failed to give positive reactions. Soon, however, discordant reports appeared. Meyer (11) tested ten streptococci against four monovalent sera obtained by immunizing rabbits with four of the strains, and found that cross agglutination occurred. Nevertheless, the organisms from purulent processes were not affected by sera immune to streptococci from the throats of scarlet fever and rheumatism patients. From these observations he concluded that agglutination reactions could be used to separate the streptococci of angina from those of septic processes. Other workers then began to report promiscuous cross agglutination between the various streptococci. Weaver (12), investigating the serum of scarlet fever patients, concluded that the agglutination reaction in this disease was in no way specific. Neufeld (13) thought that, in certain cases at least, agglutinability varied inversely with virulence. Since most of the strains that he used were capable of being made highly virulent for white mice it now seems probable that they were of the hemolytic variety. Aronson (14), investigating the value of serum in the treatment of so called streptococcus infections, concluded that a polyvalent serum was necessary on account of the failure of one streptococcus from a given disease to be agglutinated by a serum produced by immunizing with another streptococcus from the same disease. He thus argued against the constancy of streptococci as specific producers of disease. Marmorek (15) reviewed the investigations that had been made up to 1902 and concluded that all human pathogenic streptococci were the same. This view was later supported by Zoeppritz (16). Fischer (17) tested twenty-one streptococci from various pathological and normal sources against sera immune to eight representative members of the series and obtained such promiscuous cross agglutination that he decided a great multiplicity of strains existed, and that the agglutination reaction was not effective in grouping them.

From the foregoing it will be seen that the question of classification was unsettled and no plan for grouping streptococci had found wide acceptance. Two courses of procedure were open. One, the result of the failure of previous work, followed the idea that all streptococci were the same, and that the differences which the various workers had endeavored to interpret as fixed characteristics were in reality the fleeting impress of chance environment. At the same time it was asserted that these inconstant organisms produced a great variety of diseases, and to explain this varied activity the theory of elective localization was formulated. This postulates transmutations to a surprising degree of frequency, and often the agency which endows a streptococcus with affinity for the stomach, or for the heart valves, or for the joints, operates in the same focus of infection, the tonsil. Proof has been offered in the production in animals of corresponding lesions in which no factor other than the activity of streptococci is considered. Inasmuch as it is insisted that the properties of elective localization are apt to be lost in the process of isolating a streptococcus, and that the first culture even though mixed must be used, this theory in its present form eludes accurate experimental demonstration.

The other course was to attack the problem from the standpoint of biochemical reactions. Gordon (18) studied the fermentation reactions of streptococci on certain test substances. This line of work was soon taken up by others, and Andrewes and Horder (19) reported extensive studies on 1,200 strains and succeeded in placing the so called pathogenic streptococci in four groups. They found that outside of the first group called *pyogenes*, which was composed largely of streptococci from purulent processes, the source of the streptococci did not accurately determine their place in the groups. In analyzing the results of the reactions, the different groups are found to contain from 14 to 42 per cent of variants. Objections to these biochemical tests on the grounds of inconstancy were advanced by Ritchie (20), Walker (21), and others. However, the discrepancies were elicited by doing the tests under diverse conditions of age, viability, and culture media, and do not discredit the method as much as they indicate the necessity for uniformity in performing the tests. The reasons for variation have been discussed by Thro (22, 23) and Broadhurst (24). Very recently Holman (25) suggested a more extensive differentiation of the entire streptococcus group, based upon their hemolytic and methemoglobin production properties and upon their fermentation of lactose, mannite, and salicin. Sixteen groups are described, but as so few carbohydrates are employed no variants of these groups are recognizable.

Andrewes and Horder found agglutination reactions "troublesome and disappointing," but attempts were soon directed to a correlation of fermentation and agglutination reactions. Floyd and Wolbach (26) concluded that fermentation reactions could be used to separate streptococci into large groups, while agglutination reactions merely emphasized the individuality of members. Kligler (27), on the other hand, found that agglutination reactions ran strikingly parallel with fermentation reactions.

Although Schottmüller (28) in 1903 emphasized the difference between streptococci on the basis of their action upon blood agar plates, very little attention is paid to this factor in the above reports, except for Holman's work. Most workers agree in establishing a strong connection between a certain number of purulent infections and the hemolytic streptococcus (*Streptococcus pyogenes*). It is not unreasonable to assume that much of the cross agglutination reported was due to the fact that one type of streptococcus, the hemolytic, was tested, because the source of many of the strains was a purulent process. Analysis of the reports of Meyer (11) and Neufeld (13) illustrate this point. Analyzing the report of Kligler, in which attention was paid to the hemolytic property of the strains, we find support for the argument that the property of producing hemolysis is important. Kligler tested a serum produced by immunizing a rabbit against a hemolytic strain and found that it agglutinated ten out of twenty-nine hemolytic strains, none out of seventeen "green" strains, and five out of twelve indifferent strains. Thus this serum displayed a marked capacity to agglutinate hemolytic strains; a marked capacity to agglutinate indifferent strains; but no capacity to agglutinate green strains. Two sera immune to green strains agglutinated two out of twenty-nine hemolytic strains and seven out of seventeen green strains. Thus their agglutinating capacity was marked for green but weak for hemolytic strains.

These brief considerations make it logical to require that the hemolytic and non-hemolytic groups should be studied separately in an attempt to classify, and for this reason in the present work the study was limited to non-hemolytic strains alone.

EXPERIMENTAL.

In studying the organisms on which this report is based, attention was paid to the source, growth in plain broth, effect on ascitic-dextrose-agar, solubility in ox bile, action on red cells, according to the method used by Lyall (29), and virulence. In addition, the fermentation reactions were compared with the agglutination and complement fixation reactions.

Methods.

1. For testing the effect of streptococci on red cells, dilutions of a 24 hour broth culture were made in a row of small tubes, using plain broth as a diluent. Each tube contained 0.5 cc. of culture dilution, the doses being graduated as follows: the first tube, 0.5 cc. of culture; the second, 0.25 cc.; the third, 0.12 cc., etc. To each tube 0.5 cc. of 5 per cent saline suspension of sheep red blood corpuscles was added. The mixtures were read after incubation in the water bath, at 37°C. for 1 hour. Streptococci either (1) hemolyze the cells, (2) produce methemoglobin in the unhemolyzed cells, or (3) have no effect upon the cells. The method is not accurately quantitative, but the results are more nearly comparable than are those obtained with the blood agar plate method. However, for comparison both methods were employed.

2. For testing virulence, white mice were inoculated with 18 hour broth cultures in amounts of 2.0 cc., 1.0 cc., and 0.1 cc. If an organism was lethal at 0.1 cc. it was titrated further.

3. Fermentation reactions were done with litmus milk and with raffinose, inulin, salicin, and mannite as test substances. Media containing these were prepared by adding 1 per cent of the test substance to Hiss serum water. In examining the effect of the streptococci on these carbohydrates, a tube of each was inoculated with about 0.2 cc. of actively growing broth culture, incubated, and observed for 10 days.

4. For the animal immunization, a rabbit was used for each of the twenty-eight streptococci. Immunization was effected by injecting the animals intravenously with saline suspensions of killed streptococci at 4 day intervals, in doses equivalent to 10 cc. of broth culture. The injections were continued until the serum of an animal gave marked complement fixation with an antigen made from the corresponding streptococcus. Most of the animals produced good complement-fixing antibodies after six to ten injections.

The sera from the same animals were used for both complement fixation and agglutination reactions, and sera of the same or nearly the same dates were used for both reactions. In the case of complement fixations, as soon as a serum showed definite fixation with its

own antigen, it was tested on the same day with the same materials, hemolytic system, etc., against the twenty-eight antigens, and those antigens which gave fixation were noted. A few days later the animal was bled again and the serum was titrated against those antigens with which fixation was previously obtained. The above precautions were taken because it was noticed, in a few instances, that an old serum tended to give fixation with an increased number of antigens. In performing the agglutination reactions, a dextrose broth culture of a streptococcus was tested against the twenty-eight sera. The reason for this arrangement is obvious, since it was desirable to perform both tests with sera obtained at the same bleeding. All of the twenty-eight rabbits could not be immunized at the same time, and while the age of the serum does not affect agglutinating capacity, complement fixation reactions must be performed with fresh serum because variations in tests showed this to be advisable. Accordingly, while all the sera were on hand when the agglutination tests were made, owing to the uncertainty of keeping rabbits alive over a long period, complement fixation tests had to be made as soon as an animal's serum showed sufficient immunity.

5. In the complement fixation reactions various constituents were used in the following quantities: 0.1 cc. of streptococcus antigen, two units of complement and anti-sheep amboceptor, and descending amounts of immune serum, as 0.1 cc., 0.05 cc., 0.025 cc., etc. The complement-antigen-serum mixtures were made up to 1.5 cc. and incubated in the water bath, at 37°C. for 1 hour. Sensitized cells prepared by mixing 0.5 cc. of amboceptor dilution and 0.5 cc. of a 5 per cent suspension of cells were added, and the tubes again placed in the water bath at 37°C. for 1 hour before reading.

The antigens were prepared as follows. Washed sediment of a 24 hour broth culture was suspended in 5 cc. of saline, and 5 cc. of absolute alcohol were added. The resulting precipitate was separated by centrifugalization and desiccated *in vacuo*. It was then ground and weighed. 10 mg. were dissolved in 5 cc. of a 2 per cent antiformin solution in the water bath at 56°C. and the solution was neutralized with 0.1 N sulfuric acid, with litmus paper as an indicator. The free chlorine was liberated by adding one or two drops of 5 per

cent sodium thiosulfate, the end-result being tested with potassium iodide starch paper. The solution was made up to 10 cc. with carbolized saline, and centrifugalized. 1 cc. of the antigen then represented 1 mg. of dried ground precipitate. This method was always followed. It therefore seems reasonably certain that the antigens in these experiments represent a constant amount of their corresponding streptococci.

6. Agglutination reactions were performed by making dilutions of serum in 0.5 cc. of dextrose broth, so that the first tube contained 0.1 cc. of serum; the second, 0.05 cc.; the third, 0.025 cc., etc. To each tube 0.5 cc. of a 24 hour dextrose broth culture was added. The mixtures were placed in the water bath at 37°C. for 2 hours, and readings were made after they had stood in the ice box over night.

RESULTS.

Table I contains a list of the organisms, their source, and the main characteristics as outlined above. Each of the twenty-eight strains was obtained from one colony.

It will be seen that with the exception of one organism (B1), which was possibly a contamination, all may be said to have come from pathological sources. Two diseases, acute rheumatic fever and bacterial endocarditis, are chiefly represented. In the case of tonsillitis, the streptococci indicated were from the predominating colonies. All are true streptococci as can be seen from their morphology, their action on ascitic-dextrose-agar, and their insolubility in bile. Those which produce methemoglobin in the presence of red blood cells are called green. Three of the streptococci were indifferent in their action upon red cells. The group from bacterial endocarditis is striking for its lack of virulence, while the rheumatism group is consistently more virulent. Attempts to raise the virulence of a few of the streptococci by passage through white mice were unsuccessful.

Table II shows the results of the fermentation reactions. All the organisms were tested soon after isolation. Thus the original tests were not all made at the same time or with the same lot of media. The final tests were performed at the same time and with the same

TABLE I.

Streptococcus.	Source.	Appearance in broth.	Length of chains. Diplococci.	Effect on ascitic-dextrose-agar.	Solubility in bile.	Effect on red blood cells.	Lethal dose for white mice.
1 A65	Blood culture; acute arthritic rheumatism.	Turbid (diplococci).	2–6	Precipitate.	Insoluble.	Green.	0.1 cc. of 24 hr. broth culture.
2 59F	"	"	6–20	"	"	"	0.1 cc.
3 A49	"	"	6–20	"	"	"	0.1 "
4 B38	"	"	4–20	"	"	"	Not fatal at 1 cc.
5 B39	"	Semiturbid; granular sediment.	4–20	"	"	"	" " " 2 "
6 A135	"	Turbid.	2–8	"	"	"	" " " 2 "
7 38D	"	"	8–10	"	"	"	0.1 cc. of 24 hr. broth culture.
8 A141	Blood culture; rheumatic endocarditis.	"	6–20	"	"	"	2 cc.
9 A119	"	"	2–8	"	"	"	Not virulent at 2 cc.
10 A179	Culture from heart valve; P. M. rheumatic endocarditis.*	"	6–12	"	"	"	2 cc.
11 B26	Acute tonsillitis.	Coarse diplococcus; turbid.	10–30	"	"	"	Not tested.
12 B23	"	Turbid.	10–20	"	"	"	Not fatal at 2 cc.
13 K	"	"	10–20	"	"	"	0.1 cc.
14 A102	Sputum; lobar pneumonia.	Coarse diplococcus; turbid.	4–20	"	"	"	0.5 "

15	B1	Surface colony on plate; acute arthritic rheumatism.	Coarse diplococcus; turbid.	10–30	Precipitate.	Insoluble.	Green.	1 cc.
16	A140	Knee joint; P. M. chronic arthritis.	Turbid.	4–10	"	"	"	0.1 cc.
17	A84	Blood culture; P. M. bacterial endocarditis.	Coarse diplococcus; turbid.	4–20	"	"	"	0.5 "
18	A4	Blood culture; A. M. bacterial endocarditis.	Turbid.	6–20	"	"	"	Not fatal at 2 cc.
19	B4	"	"	2–8	"	"	"	"
20	A148	"	"	2–8	"	"	"	"
21	XK	"	"	2–8	"	"	"	"
22	A30	"	"	10–50	"	"	"	"
23	R	"	"	4–10	"	"	"	"
24	B29	"	"	2–6	"	"	"	"
25	O	"	Clear medium, soft sediment.	10–30	"	"	"	"
26	A26	"	Diplococci, bacillary shapes; semiturbid; clumps.		"	"	Indifferent.	"
27	A56	"	Clear, clumps; granular sediment.	2–6	"	"	"	"
28	MB	"	Clear; granular sediment.	2–6	"	"	"	"

*P. M. indicates postmortem; A. M., antemortem.

TABLE II.

Streptococcus.		Interval between 1st and 2nd tests.	No. of generations between 1st and 2nd tests.	Fermentation reactions.				
				Milk.	Raffinose.	Inulin.	Salicin.	Mannite.
		mos.						
1	A4	19	10	+*	−	−	−	−
2	O	15	4	+	−	−	−	−
3	XK	8	5	+	−	−	−	−
4	59F	30	15	+	−	−	−	−
5	A49	18	4	+	−	−	−	−
6	A141	15	5	+	−	−	−	−
7	A179	14	5	+	−	−	−	−
8	B39		One test.	+	−	−	−	−
9	A30	18	10	+	+	−	−	−
10	R	13	7	+	+	−	−	−
11	A65	16	5	+	+	−	−	−
12	B29	5	2	+	+	−	−	−
13	A148	14	5	+ +	− +	+ −	+ −	− −
14	A102	15	4	+	+	−	+	−
15	B38		One test.	+	+	+	−	−
16	B23	5	3	+ +	− +	+ +	+ +	+ −
17	K†	15	6	+ +	+ −	− −	+ +	− −
18	MB	8	4	+	−	−	+	−
19	38D			+	−	−	+	−
20	B26	5	8	+	−	−	+	−
21	A140	15	5	+ +	− −	+ −	+ +	− −
22	A119	16	6	+	−	+	+	−
23	B4	8	2	+	−	+	+	−
24	B1	8	7	−	−	+	+	−
25	A26	18	9	+	−	−	+	+
26	A135	16	6	+	−	−	+	+
27	A56	19	8	+	+	+	−	+
28	A84			+ +	+ −	− −	+ +	− +

*+ indicates acid and clot; —, no acid or clot.

† The second test of No. 17 K is probably incorrect as it was recently found that the original Strain K was lost.

media. This may be an important factor in interpreting variations. The arrangement followed in the table is based on the final tests. If an organism showed a variation on second test, the test was done

three times. In five instances, the first and second tests varied. Accordingly in these five the first reading represents one test, and the second reading represents three tests.

It will be seen that all the strains can be placed in two groups of the Andrewes and Horder classification; namely, the *fecalis* group, the five mannite fermenters, and the *salivarius* group which embraces the remaining twenty-three. The results agree with those of the above mentioned investigators in showing that the source is not a determining factor in the grouping. These streptococci could be subdivided into more groups than merely those of *salivarius* and *fecalis*, if the classification advised by Holman could be applied. But this requires testing the fermentation capacities of the various strains with lactose. This was not done. No general conclusions can be drawn from this table. It merely shows that most of the organisms belong to a group called *salivarius*, the feature of which is the fermentation of raffinose but which is permitted to contain variants that do not ferment this substance. With a series of only twenty-eight members a grouping on the basis of fermentation is not conclusive since we have to place members of widely varying fermentative activity together, in a grouping which investigation on a larger scale shows to be logical.

The results of the complement fixation reactions are striking. In the early part of this experiment, the sera tested showed a wide variation in fixing capacity. Thus, while one serum would fix only its own antigen, another would fix many more, and the results seemed accidental and confused. As the work proceeded, however, it became evident, first, that, in general, where a serum gave extensive cross fixation, its corresponding antigen was fixed by very few sera, and then only by sera of equally wide fixing capacity; and second, though to a less noticeable degree, that the sera which gave fixation with a small number of antigens corresponded to antigens which gave fixation with a large number of sera. In other words, there was an inverse ratio between the fixing capacity on the part of a serum and the capacity to be fixed on the part of the corresponding antigen. Inspection of Table III will make this clear. Serum 3 has wide fixing capacity; Antigen 3 is fixed only by sera which are nearly identical in fixing capacity with Serum 3. Again, Serum 28 gives exten-

sive fixation but Antigen 28 is fixed only by Serum 28. On the other hand, Sera 8, 11, 14, and 20 are very limited in fixing capacity; the corresponding antigens, however, are fixed by many sera.

This phenomenon occurred with sufficient regularity to constitute a fact. It is important to determine its cause. The explanation adopted was based on a consideration of the complexity of the different streptococci. It was thought that some streptococci might be more complex than others, perhaps in the structure of their chemical nucleus. If a rabbit is injected with such a streptococcus, its serum will present an antibody capable of fixing this streptococcus, and such other streptococci as contain the same or some of the same structural units. But if a rabbit is injected with an organism containing only one of these units, it will present an antibody incapable of union with the more complex structures and will fix only streptococci of corresponding simplicity. This led to the placing of some antigens above others in the table, and to the formation of subgroups.

Table III illustrates these points. Sera 3 to 7 have large fixing capacity. But Antigens 3 to 7 are fixed only by sera which have nearly equal fixing capacity: *viz.*, Nos. 3 to 7. This for the time being is called Group I. Besides fixing its own antigens this group of sera fixes Antigens 8 to 16. Of these, Nos. 8 to 12 may be said to constitute a separate group, first, because Antigens 8 to 12 are fixed by Sera 3 to 7, and second, because Sera 8 to 12 show similar though not identical relationships with antigens. Antigens 13 to 16 as well as Sera 13 to 16 show new relationships, and constitute an intermediate group, because by antigen and by antibody they are related to Groups I and II. Inspection of the table will show that these connections are irregular, but attention must be paid to these subgroups, because in a larger series of organisms such subgroups may assume large proportions. In a small series like the present one, irregular members in the intermediate positions are to be expected. Antigens 21 to 28 as well as the corresponding sera seem to constitute a group independent of Group I which is called Group II. In this group also tendency to subgroup formation can be readily seen, and no doubt, in a larger series, Nos. 22, 24, and 26 might be the heads of a number of identical organisms. In fact, No. 28 is the

only serum of sufficiently wide reacting capacity to deserve an independent grouping in the way that Sera 3 to 7 are distinct. Nos. 17 to 20 are called Group IIa because they are closely related to Group II, both by antigen and antibody, and are intermediate members because of their relations with Groups Ia and Ib. Finally Group II is seen to be connected with Group I through Nos. 1 and 2. The naming of groups is arbitrary and merely for the purpose of distinction.

The complement fixation reactions on which this grouping is based showed some variation, where the tests were repeated; but the variations that occurred were such as not to affect the grouping of the streptococcus in question. Thus Serum 11 fixed Antigens 11 and 12, while the serum of another rabbit immune to No. 11 fixed Antigens 10, 11, and 12. In like manner, two rabbits were immune to Streptococcus 3 and their sera differed only in the intensity of fixation with Antigens 11, 13, and 21. Such variations may be incidental to uncontrollable factors in the complement fixation reaction.

The sera of twelve of the twenty-eight rabbits, including Nos. 2 and 21, were found to give negative reactions before immunization was begun and hence the non-specific fixation sometimes ascribed to normal rabbit serum cannot be a factor in these tests.

It will be noted that while an antigen is usually fixed more completely by its homologous serum, the rule does not always apply, as for example, Strain 22. The same also applies to the action of sera. Some rabbits yielded sera of weak fixing power, and one, No. 18, yielded serum which gave agglutination but not complement fixation reactions.

In the table agglutination reactions outside the zone of complement fixation are indicated by an A.

Table IV shows the results of the agglutination reactions, which were less satisfactory. A strongly positive reaction was not always present between serum and the corresponding streptococcus, and cross reactions were fewer. Agglutination occurred in some cases where there had been no complement fixation reaction, but, as Table III indicates, this apparent discrepancy does not interfere with the grouping effected by complement fixation reactions.

TABLE III.

Results of Complement Fixation Reactions.

Rabbit serum.	Streptococcus antigens.																										
	1 A4	2 A26	3 K	4 A56	5 MB	6 A84	7 A140	8 38 D	9 A102	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135	17 XK	18 A30	19 A179	20 R	21 A65	22 59F	23 A49	24 O	25 B29	26 B38	27 B39	28 B26
1 A4	4 4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
2 A26	4 4 4	4 4 4	o	o	4 3 1	3 3 2	1 1 o	o	o	o	o	o	o	3 2 1	o	o	o	o	o	o	o	o	o	o	o	o	o	o
3 K	o	3 3 2	4 4 4	4 4 4	3 4 4	3 3 3	4 2 o	4 4 4	4 4 4	4 4 4	4 2 2	4 4 4	3 3 2	4 4 3	4 4 3	o	o	o	o	o	o	o	o	o	o	o	o	2 1 o
4 A56	o	2 1 o	2 1 o	4 4 4	4 4 4	3 1 o	4 4 2	4 4 1	4 3 1	4 4 1	4 4 4	4 4 3	3 3 1	4 4 3	4 4 3	4 3 2	o	o	o	o	o	o	o	o	o	o	o	o
5 MB	o	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	1 1 o	4 4 4	4 4 4	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o
6 A84	o	3 1 o	3 1 o	4 4 1	4 4 1	4 4 4	4 3 1	4 3 1	4 3 1	4 3 1	4 3 1	4 3 1	o	4 3 1	3 2 1	o	o	o	o	o	o	o	o	o	o	o	o	o
7 A140	o	o	3 o o	4 4 4	3 o o	o	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	o	3 o o	3 o o	o	o	o	o	o	o	o A	o A	o	o	o	o	o
8 38D	o	o	o	o	o	o	o	4 4 4	o	o	o	o	o	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o
9 A102	o	o	o	o	o	o	o	4 4 2	4 4 4	4 4 4	4 4 4	4 4 4	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o	o
10 A119	o	o	o	o	o	o	o	4 1 o	4 1 o	4 3 3	4 4 2	4 4 2	o	o A	o A	o	o	o	o	o	o	o	o	o	o	o	o	o
11 B4	o	o	o	o	o	o	o	o	o	o A	4 4 4	2 2 2	o	o A	o A	o A	o	o	o	o A	o	o	o	o	o	o	o	o
12 A148	o	o	o	o	o	o	o	o	o	2 3 2	4 4 1	4 4 2	3 2 o	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o
13 B1	o	o	o	o	o	o	o	4 4 3	1 4 4	1 3 2	1 1 1	2 3 2	4 4 4	o	o	o	o	o	o	4 4 4	o	o	o	o	o	o	o	o

14 B23	o	o	o	o	o	o	o	o	2 3 1	o	o	o	o	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	o A	o
15 A141	o	o	o	o	o	o	o	4 4 4	o	4 4 4	3 1 o	4 4 4	o	o	4 4 4	o	o	o	o	o	o	4 4 3	4 4 o	o A	o	o	o	o
16 A135	o	o	o	o	o	o	o	o	o	o	o A	o A	o	o	o	4 4 4	o	o	o	o A	o	o	o A	o	o	o A	o	o
17 XK	o	o	o	o	o	o	o	o	o	o	3 2 o	4 2 o	o	4 3 1	o	o	4 3 2	o	o	4 3 2	4 2 1	o	o	o	o	o	o	o
18 A30	o	o	o	o	o	o	o	o	o	o A	o A	o A	o	o	o A	o	o	o A	o	o A	o A	o	o	o	o A	o A	o	o
19 A179	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o A	o	o	o	4 4 4	o	o	o A	o A	o	o	o	o A	o
20 R	o	o	o	o	o	o	o	o	o	o	o A	o	o A	o	o	o	o	o A	o	4 4 4	o	o	o	o	o	o	o	o
21 A65	o	o	o	o	o	o	o	o	o	o	o A	o	o	o	o	o	o	o A	o	o A	4 4 1	o	o	o	o	o	o	o
22 59F	o	o	o	o	o	o	o	o A	o	o	o	o	o	o	o A	o	o	o	o	o	o	4 4 4	4 4 4	o	o	o	o	o
23 A49	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	o	o A	o	o	o	4 4 4	4 4 4	o	o	2 2 o	o	o
24 O	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	o	o	o	4 4 4	o	o	o	4 4 4	1 2 4	4 4 4	3 4 4	o
25 B29	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o	4 4 4	o	o	o	4 4 4	4 4 4	4 4 4	4 4 4	o
26 B38	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o	o	4 4 4	o	o	o	o	o	4 4 4	2 2 1	o
27 B39	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	4 4 4	o	4 3 2	4 4 4	o	4 2 1	1 1 o	o	o	4 4 2	4 4 1	o
28 B26	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	4 4 3	o A	4 4 3	4 4 3	4 4 3	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 3	4 4 4

4 = complete fixation. 3 = 25 per cent of cells hemolyzed. 2 = 50 per cent of cells hemolyzed. 1 = 75 per cent of cells hemolyzed. o = no fixation. $\overset{o}{A}$ = agglutination but no fixation.

The numbers indicate plus signs, and the reading from above downward indicates the following dilutions: the top figure in each group, a dilution of 0.1 cc.; the second figure, 0.05 cc.; the third, 0.025 cc. Only three readings are inserted on account of lack of space. Five dilutions were used in each test.

TABLE IV.

Results of Agglutination Reactions.

Rabbit serum.		Streptococci.																										
		1 A4	2 A26	3 K	4 A56	5 MB	6 A84	7 A140	8 38D	9 A102	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135	17 XK	18 A30	19 A179	20 R	21 A65	22 59F	23 A49	24 O	25 B29	26 B38	27 B39	28 B26
1	A4	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	A26	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	K	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	A56	−	−	−	+	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
5	MB	−	−	−	+	+	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
6	A84	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	A140	−	−	−	−	−	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−
8	38D	−	−	−	−	−	−	−	+	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
9	A102	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	A119	−	−	−	−	−	−	−	−	−	+	+	+	−	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−
11	B4	−	−	−	−	−	−	−	−	−	+	+	+	−	+	±	±	−	−	−	+	−	−	−	−	−	−	−	−
12	A148	−	−	−	−	−	−	−	−	−	−	+	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
13	B1	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	B23	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
15	A141	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−
16	A135	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	−	+	−	−	+	−	−
17	XK	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
18	A30	−	−	−	−	−	−	−	−	−	+	+	+	−	−	±	−	+	−	+	−	+	−	−	−	+	+	−	−
19	A179	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	+	−	−	+	+	−	−	−	+	−
20	R	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	±	−	−	−	−	−	−	−
21	A65	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−
22	59F	−	−	−	−	−	−	−	±	−	−	−	−	−	−	±	−	−	−	−	−	−	+	+	−	−	−	+	−
23	A49	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	+	−	−	−	+	+	−	−	−	−	−
24	O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	+	−	−	−	−
25	B29	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
26	B38	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
27	B39	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	±	−	−	−	+	−	−	−	+	+
28	B26	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	+	−	−	−	−	−	−	−	+

+ indicates marked agglutination; ±, moderate agglutination; −, no agglutination; o, test not done.

If greater reliance could be placed in the results of the agglutination tests, contrast could be pointed out between the form of the chart of agglutination reactions, and that of complement fixation reactions. This is especially noticeable in the upper left-hand corner where sera with wide fixing capacity have very narrow agglutinating capacity. But it is better to await a similar finding in the study of other groups of bacteria, as well as more definite knowledge concerning the mechanism of the two reactions in question.

It will be noticed that there is no agreement between the grouping based on fermentation reactions and that based on agglutination reactions. For example, Strains 38D and B26 ferment the test substances in the same way; yet they occupy widely separated places in the chart of agglutinations. The same can be said of Strains A26 and A135 as well as of A65 and B29. It is probable that fermentation tests and agglutination tests are instruments which measure two widely different activities of streptococci; so that classifications based on these two tests have little common ground for comparison.

DISCUSSION.

The simplest observation that may be made on examining the chart of complement fixation reactions is that there is a right-sided element and a left-sided element, together with a group in the mid-zone, where these elements mingle. In any arrangement of the chart, Serum 3 will always occupy a position on the side opposite the side on which Serum 28 will be. While it is true that study of more streptococci might reveal strains different from those herein tabulated, it is not probable that the general form of the chart would be changed, because there are at present no elements suggestive of a new kind of variation which further study would accentuate. A repetition of this entire experiment with the same streptococci would probably not alter the form of the chart in any way because similar reactions were found in those cases where the immunization was repeated with fresh animals and the tests were done a second time.

The next observation to be made, and one which was made first in the present work, is that the strains seem to differ in complexity

with corresponding difference in their antisera. Thus a strain which produces an antiserum of wide reacting capacity, will not itself react with a serum of narrow capacity; and a strain which produces an antiserum of narrow reacting capacity will usually react with many sera of wider reacting capacity than its own. This is the inverse ratio described above.

The work of others with other kinds of bacteria, while not expressing it directly, can be interpreted in such a way as to support both these observations. This should be the subject of another discussion. It will suffice to say that classifications of gonococci and typhoid bacilli can be arranged to show both the tendency to right-sided and left-sided features as well as the inverse ratio referred to above. Previous classifications of streptococci have not embraced more than twelve strains with corresponding antisera.

The explanation of these observations must be theoretical because the mechanism of the complement fixation reaction has not been completely determined.

If, hypothetically, we regard this reaction as an adsorption phenomenon (30), depending on differences in surface charge as well as on chemical surface configuration (31), for the combination of its different factors, it may be possible to refer to the strains on the left side of the chart as being composed of protein molecules of opposite charge to those on the right side. The complex strains would be said to be made up of several kinds of molecules of a certain charge; the simple ones of only one or two molecules of the same charge. The mid-zone would contain those strains which partake of both features. If the antisera be thought of as containing proteins of a charge opposite to that of their corresponding antigens, the failure to react with antigens of different charge can be understood. Finally, the antiserum for a complex antigen would present a complex surface configuration, while that for a simple antigen would show a corresponding simplicity.

In this way the chart of complement fixation reactions might be regarded as outlining the sphere of variation of the non-hemolytic streptococcus with regard to this one reaction.

CONCLUSIONS.

1. No connection can be demonstrated between grouping of non-hemolytic streptococci based upon fermentation reactions, and grouping based upon immunological reactions.

2. A classification of non-hemolytic streptococci can be effected by studying the complement fixation reactions between the streptococci and their antisera.

3. The arrangement of the streptococci in such a classification depends on the fact that two diverse elements are present in the group. Some strains partake entirely of one of these elements, some entirely of the other, while other strains partake of both.

4. The arrangement is further determined by the fact that among the strains composed of one element there are differences in complexity, some strains being made up of many molecules or features, others having much simpler structure. This gives rise to an inverse ratio between the fixing capacity of a serum and the capacity of the corresponding antigen to be fixed.

BIBLIOGRAPHY.

1. Fehleisen, F., Die Aetiologie des Erysipels, Berlin, 1883. Quoted in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, iv, 454.
2. von Lingelsheim, W., *Z. Hyg.*, 1891, x, 331. Quoted by Winslow, C.-E. A., and Winslow, A. R., The Systematic Relationships of the Coccaceæ, New York, 1908.
3. Kurth, H., *Arb. k. Gsndhtsamte.*, 1891, vii, 389. Quoted in Kolle and von Wassermann, Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, iv, 456.
4. Rosenbach, A. J. F., Mikro-Organismen bei den Wund-Infections-Krankheiten des Menschen, Wiesbaden, 1884.
5. Van de Welde, H., *Arch. méd. exp. et anat. path.*, 1897, ix, 835.
6. Van de Welde, *Centr. Bakteriol., Ite Abt.*, 1898, xxiii, 481.
7. Baginsky, A., and Sommerfeld, P., *Berl. klin. Woch.*, 1900, xxxvii, 588.
8. Hasenknopf and Salge, *Jahrb. Kinderh.*, 1903, lviii, 218.
9. Moser, P., and von Pirquet, C. F., *Centr. Bakteriol., Ite Abt., Orig.*, 1903, xxxiv, 560.
10. De Wacle, H., and Sugg, E., *Arch. internat. pharm. et thérap.*, 1904, xii, 205.
11. Meyer, F., *Deutsch. med. Woch.*, 1902, xxviii, 751.
12. Weaver, G. H., *J. Infect. Dis.*, 1904, i, 91.

13. Neufeld, F., *Z. Hyg.*, 1902, xl, 54.
14. Aronson, H., *Deutsch. med. Woch.*, 1903, xxix, 439.
15. Marmorek, A., *Berl. klin. Woch.*, 1902, xxxix, 299.
16. Zoeppritz, B., *Med. klin.*, 1909, v, 1112.
17. Fischer, H., *Centr. Bakteriol., 1te Abt., Orig.*, 1904, xxxvii, 449.
18. Gordon, M. H., *Lancet*, 1905, ii, 1400.
19. Andrewes, F. W., and Horder, T. J., *Lancet*, 1906, ii, 708.
20. Ritchie, J., *Lancet*, 1908, ii, 374.
21. Walker, E. W. A., *J. Path. and Bacteriol.*, 1912–13, xvii, 140.
22. Thro, W. C., *J. Infect. Dis.*, 1914, xv, 234.
23. Thro, *J. Infect. Dis.*, 1915, xvii, 227.
24. Broadhurst, J., *J. Infect. Dis.*, 1915, xvii, 277.
25. Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377.
26. Floyd, C., and Wolbach, S. B., *J. Med. Research*, 1913–14, xxix, 493.
27. Kligler, I. J., *J. Infect. Dis.*, 1915, xvi, 327.
28. Schottmüller, H., *Münch. med. Woch.*, 1903, l, 849, 909.
29. Lyall, H. W., *J. Med. Research*, 1914, xxx, 487, 515.
30. Bayliss, W. M., Principles of General Physiology, New York, 1915, 107.
31. Ostromuislenski, I. I., *J. Russ. Phys. Chem. Soc.*, 1915, xlvii, 263; *Chem. Abstr.*, 1916, x, 214.

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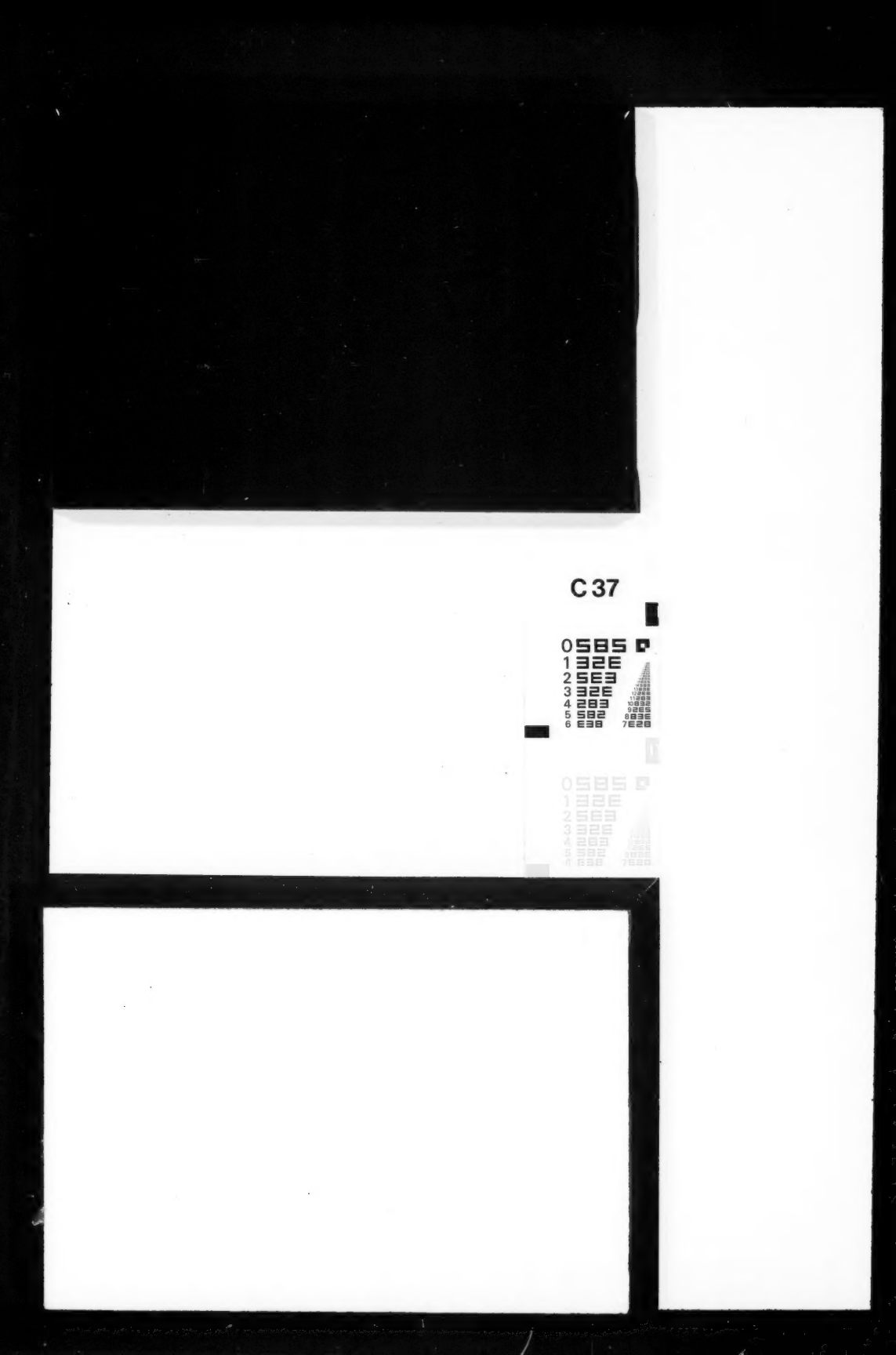
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